The role of PAK4 in pancreatic cancer

King, Helen

Awarding institution:
King's College London

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Helen King

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Division of Cancer Studies
King's College London

Declaration of Authorship

I declare the work presented in this thesis is my own, with contributions from others properly cited and acknowledged. This work was performed between October 2011 and October 2014 in the Division of Cancer Studies, King's College London.

Helen King

October 2014

Acknowledgements

This is for my nana, Joan, who sadly passed away during the final phases of writing this thesis. Without your words of “encouragement” I would never have thought it possible for me to go back to university at the age of 23 and get through these last seven years. You were the best nana anyone could wish for, you said it how it was, but you always believed in me. I know this achievement would have made you so proud and I am so sad that you didn’t get to see me finish, so I dedicate this thesis in your loving memory.

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is highly aggressive. It is one of the most lethal solid malignancies and has a 5-year survival rate of less the 3%. There is a high incidence of K-ras mutations in pancreatic cancer and it also thought that p21-activated kinase 4 (PAK4) is amplified in pancreatic cancer tissue. Studies show that PAK4 was not found in a mutated, oncogenic form but rather that its activation was likely to be due to mutations in the K-ras gene. It has consequently been hypothesised that K-ras gene mutations combined with PAK4 gene amplification could increase ras activity within pancreatic cancer.

PAK4 is a member of the PAK family of serine/threonine kinases which act as effectors for several small GTPases. They are involved in a wide range of signalling pathways including cell motility, survival and proliferation; therefore, abnormal PAK signalling can contribute to a number of disease states. In particular, PAK4 is oncogenic when overexpressed, promoting cell survival, migration and anchorage-independent growth. While the mode of PAK4 regulation is not well understood, there is evidence from our lab, and others, that PAK4 may lie within a phosphatidylinositol-3-Kinase (PI3K) pathway, which is known to be downstream of RAS. This pathway can be activated via hepatocyte growth factor (HGF), which signals through the receptor tyrosine kinase, c-Met. c-Met acts as a proto-oncogene and increased expression of both c-Met and circulating HGF have been observed within pancreatic cancer. Such data suggest the potential existence of a c-Met/RAS/PI3K/PAK4 signalling module. However, a direct *in cellulo*

relationship between PAK4 and RAS has not been reported and the nature of the relationship between PAK4 and PI3K remains to be fully elucidated.

I have established that PAK4 expression is elevated in pancreatic cancer cell lines and correlated this with c-Met, K-ras and the p85α subunit of PI3K expression. Furthermore, I have validated a novel interaction between PAK4, K-ras and p85α. Work to date suggests that the PAK4:p85α interaction may be dependent on the scaffold protein Gab1. Moreover, I have found that in PAK4 deficient cells there is a reduction in the phosphorylation of Akt, a PI3K pathway target, further supporting the hypothesis that PAK4 functions downstream of PI3K. To complement my biochemical studies, I have established that PAK4 knockdown leads to decreased cell migration in 2D and demonstrated that this is phenocopied by pharmacological inhibition of PI3K. Subsequently, I optimised a pancreatic cancer organotypic model for HGF-mediated invasion and used this model to demonstrate that PAK4 deficient cells have reduced invasive potential. These results implicate a role for PAK4 within the RAS/PI3K pathway, which is highly upregulated within pancreatic cancer and therefore suggest that PAK4 could be a key player in pancreatic cancer disease progression.

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Abbreviations

ADM	Acinar to ductal metaplasia
AID	Autoinhibitory domain
Ang-2	Angiopoietin-2
Arp2/3	Actin related protein 2/3
Arx	Aristaless-related homeobox
bHLH	Basic helix-loop-helix
BOP	Nitrosamine *N*-nitrosobis-(2-oxopropyl)amin
Cdc42	Cell division control protein-42
CDK5RAP3	CDK5 kinase regulatory subunit-associated protein 3
CRIB	Cdc42/Rac interactive binding region
CSC	Cancer stem cell
CTGF	Connective tissue growth factor
DEspR	Dual endothelin1/VEGF signal peptide receptor
DR	Desmoplastic reaction
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
FGF	Fibroblast growth factor
GAP	GTPase activating protein
GBD	p21-GTPase binding domain
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GEMM	Genetically engineered mouse models
GID	GEH-H1/Gab1-interacting domain
GTP	Guanosine triphosphate
HMGA1	High-mobility group A1
Hnf6	Hepatocyte nuclear factor 6
IL-6	Interleukin-6
IPMN	Intraductal papillary mucinous neoplasm
JNK	c-Jun N-terminal Kinase
kDa	Kilodalton
KGF	Keratinocyte growth factor
LIMK	LIM-kinase

LSL	Lox-Stop-Lox
MAPK	Mitogen activated protein kinase
MCN	Mucinous cystic neoplasm
MIC	Metastatic-initiating cell
MLC-2	Myosin light chain-2
MMP	Matrix metalloproteinase
mTORC2	Mammalian target of rapamycin complex-2
Neurog3	Neurogenin3+
Nkx2.2	NK2 homeobox 2
NMR	Nuclear magnetic resonance
NmU	Neuromedin U
Np-1	Neuropilin-1
NRP-2	Neuropilin-2
PAKs	p21-activated kinases
PanIN	Pancreatic intraepithelial neoplasia
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet derived growth factor
PDK-1	3-phosphoinositide-dependent protein kinase-1
Pdx1	Pancreatic and duodenal homeobox 1
PH	Pleckstrin homology
PI-4,5-P_2	Phosphatidylinositol-4,5-bisphosphate
PI3K	Phosphatidylinositol 3-kinase
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PIX	PAK-interacting exchange factor
PKB	Protein kinase B
PKD	Protein kinase D
PP	Pancreatic polypeptide
PSC	Pancreatic stellate cell
PTEN	Phosphate and tensin homolog
Ptf1A	Pancreas specific transcription factor 1A
RTK	Receptor tyrosine kinase
SHH	Sonic Hedghog
SMA	Smooth muscle actin

SOS	Son of sevenless
SSH-1L	Sling-Shot phosphatase-1L
STAT3	Signal transducer and activator of transcription 3
TGF-β	Transforming growth factor-beta
VEGF	Vascular endothelial growth factor
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family verprolin homologous protein
YAP1	Yes-associated protein-1

Chapter 1
Introduction

Chapter 1: Introduction

1.1 Pancreatic Cancer

1.1.1 Pancreatic cancer – the disease

Pancreatic cancer is one of the most lethal solid malignancies; it is the eighth most common cancer in the UK and fourth most common cause of cancer death in higher-income countries (Kocher and Alrawashdeh, 2010). The five-year survival rate of patients diagnosed with pancreatic cancer is less than 5%, with a median survival of less than six months (Bardeesy and DePinho, 2002). These figures have shown little improvement over the last forty years, making a diagnosis of pancreatic cancer carrying one of the most dismal prognoses in medicine and highlighting the necessity for increased knowledge and novel therapeutics for the diagnosis and treatment of this disease.

The prognosis of pancreatic cancer is related to a number of factors such as clinical stage at presentation, involvement of lymph nodes and/or formation of metastases, tumour size and differentiation (Gobbi et al., 2013). One of the factors that leads to low survival rates is that pancreatic cancer often lacks specific symptoms, especially during the early phases of the disease. However, symptoms can include back pain, vague abdominal symptoms and weight loss. The majority of patients are diagnosed after presenting with obstructive jaundice, which is usually caused by obstruction of the bile duct (Freelove and Walling, 2006). For a small minority of patients (15-20%), complete surgical resection (pancreaticduodenectomy/Kausch-Whipple procedure or pylorus-preserving pancreaticduodenectomy/Traverso-

Longmire procedure) offers the only hope for cure and can improve the five-year survival rate to approximately 20% (Collins and Bloomston, 2009; Freelove and Walling, 2006; Kocher and Alrawashdeh, 2010).

There are only a small number of surgical candidates because more than half of patients have already developed distant metastases at the time of presentation. In addition, poor survival rates can be accounted to aggressive local invasion, particularly of major vessels, and resistance to chemotherapies (Bardeesy and DePinho, 2002; Collins and Bloomston, 2009; Freelove and Walling, 2006).

1.1.2 Anatomy of the pancreas

The mature pancreas contains three main cell lineages and consists of two distinctive functional units, which differ in the specific biological roles they participate in; the exocrine portion is important in digestion, and the endocrine portion plays a significant role in glucose metabolism (Pandol, 2010). The majority of the pancreas is exocrine tissue and contains two different cell types; acinar (exocrine cells) and epithelial duct cells (MacDonald et al., 2010; Mastracci et al., 2011). Acinar cells are organised into small glands called acini (see **figure 1.1A**) and are responsible for the synthesis and secretion of digestive enzymes such as trypsin, elastase and amylase (Hezel et al., 2006; Mastracci et al., 2011), which are stored as inactive enzyme forms in zymogen granules (Jura et al., 2005). The duct epithelial cells form branching networks, with acini arranged at each terminus, and produce mucous and bicarbonate. In addition, terminal end

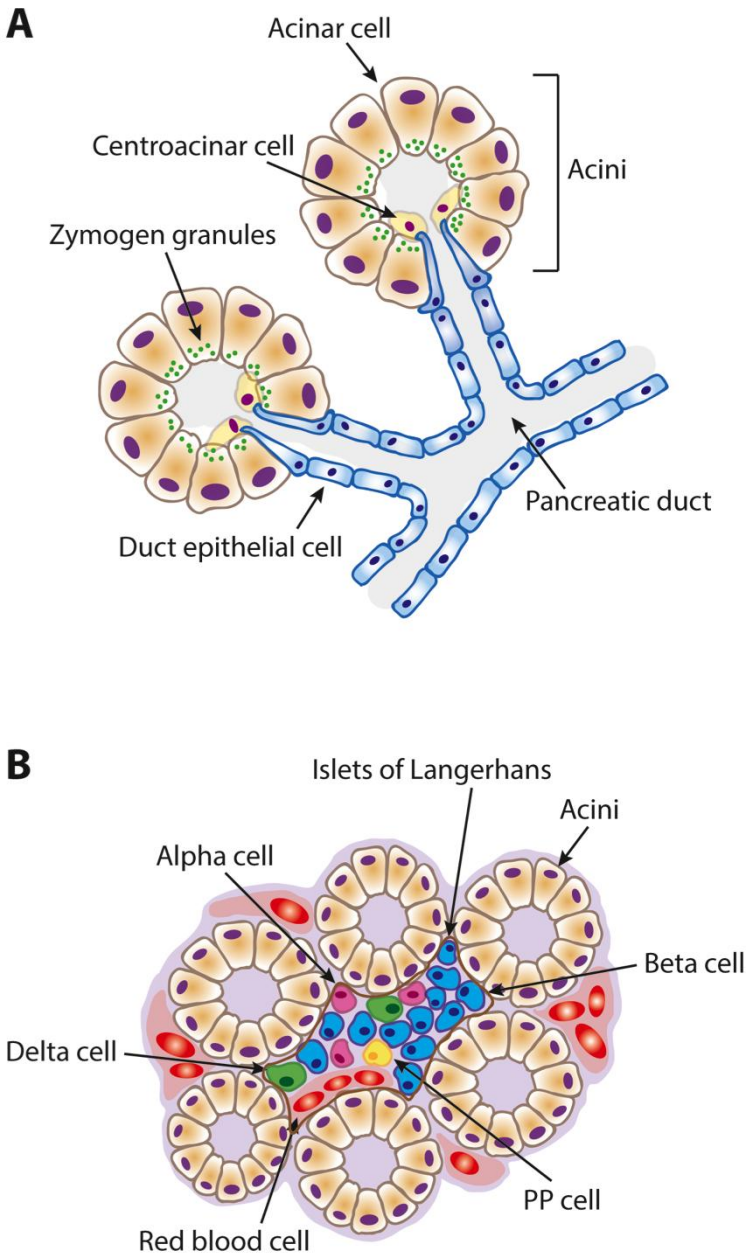


Figure 1.1: Anatomy of the pancreas. (A) Representation of an acinar unit and its relation to the pancreatic ducts. Acini form at the terminal ends of the branching ductal network and contain inactive enzymes in the form of zymogens. Also shown are the centroacinar cells, which are at the junction of the ducts and acini. (B) Pancreatic islet, which is the endocrine portion of the pancreas and is embedded in the exocrine tissue. Shown are the four main endocrine cells, which are responsible for hormone production, controlling glucose metabolism. Figure was adapted from: (Bardeesy and DePinho, 2002; Hezel et al., 2006).

duct cells are called centroacinar cells and these interface with acini cells (Reichert and Rustgi, 2011).

There are five specialised types of endocrine cells; these are organised into compact regions called the Islets of Langerhans, which are interspersed throughout the exocrine tissue (see **figure 1.1B**) (Bardeesy and DePinho, 2002; Hezel et al., 2006). The alpha and beta cells play a significant role in glucose metabolism and produce glucagon and insulin, respectively, which they release directly into the blood stream. The pancreatic polypeptide (PP), delta and epsilon cells modulate the secretory behaviour of the alpha and beta cells. They do so through expressing pancreatic polypeptide, somatostatin and ghrelin, respectively (Gradwohl et al., 2000; Prado et al., 2004).

1.1.3 Environmental and genetic risk factors

Several personal and environmental factors have been associated with the development of pancreatic cancer, with smoking being the most well established risk factor (Hassan et al., 2007). It has been reported that smokers have approximately a 1.74-fold increased risk of developing pancreatic cancer, with nitrosamines found in cigarette smoke being potent carcinogens; despite these also being present in second-hand smoke, there is no increased risk observed as yet to non-smokers exposed to environmental tobacco smoke (Iodice et al., 2008; Zhou et al., 2012). However, pancreatic cancer is generally considered a disease of the aging and is rarely seen before the age of 40, with a 40-fold increased risk by the

age of 80 (Bardeesy and DePinho, 2002) and the highest incidence rates occurring between the ages of 65-75 (Hariharan et al., 2008; Lowenfels and Maisonneuve, 2006). In addition, excessive alcohol consumption and obesity also appear to confer an increased risk (Hezel et al., 2006; Jura et al., 2005). It has also been reported that those with a benign disease of the pancreas, such as chronic pancreatitis or diabetes are also at an increased risk of developing pancreatic cancer (Hezel et al., 2006; Jura et al., 2005; Lowenfels and Maisonneuve, 2006). In chronic pancreatitis, there is increased cell turnover and proliferation as well as defective DNA repair. It has also been observed that a loss of p16 expression and KRAS mutations occur frequently in chronic pancreatitis and these are found in the high majority of pancreatic cancers (Lohr et al., 2005; Lowenfels and Maisonneuve, 2006; Rosty et al., 2003). In addition, diabetes mellitus is also considered a risk factor of pancreatic cancer and diabetes mellitus is considered both an early manifestation and an etiologic factor of pancreatic cancer (Ben et al., 2011; Everhart and Wright, 1995).

There are several germline mutations that are associated with pancreatic cancer, but only approximately 10% are thought to be caused by an inherited disorder; the most common inherited genetic disorder is a mutation in BRCA2, which can increase the frequency of pancreatic cancer (Couch et al., 2007; Lowenfels and Maisonneuve, 2006). There are also a number of familial conditions, which can be linked to an increased risk of the development of pancreatic cancer. These include; familial atypical mole-multiple melanoma (FAMMM), Peutz-Jeghers syndrome (PJS),

familial/hereditary pancreatitis, cystic fibrosis, Lynch syndrome, familial breast/ovarian cancer, Li-Fraumeni syndrome, familial adenomatous polyposis (FAP), multiple endocrine neoplasia (MEN) and Von Hippel-Lindau syndrome (VHL) (Bartsch et al., 2012; Fill et al., 1979; Hough et al., 1994; Landi, 2009) and are summarised in **table 1.1**.

Condition	Genes Involved	Risk of PDAC (%)
FAMMM	CDKN2A, CDK4	17
PJS	LKB1, STK11	36
Pancreatitis	PRSS1, SPINK1	40
Cystic fibrosis	CFTR	<5
Lynch syndrome	MLH1, MSH2	<5
Familial breast/ovarian cancer	BRCA1, BRCA2	3-8
Li-Fraumeni syndrome	TP53	<5
FAP	APC	<5
Von Hippel-Lindau	VHL	<5

Table 1.1: Familial conditions linked with an increased risk of pancreatic cancer. Table adapted from: (Bartsch et al., 2012; Ghaneh et al., 2007; Landi, 2009)

1.1.4 Cellular origins of pancreatic cancer

Pancreatic cancer can arise in different areas of the pancreas. The most commonly afflicted area is within the head of the pancreas, with approximately 65% of pancreatic cancers arising in this area. However, 15%

originate in the body of the pancreas, 10% are located within the tail and 10% are multifocal (Ghaneh et al., 2007).

There are several different tumour types that can develop in the pancreas; their classification is defined by the histological and molecular resemblance to the different pancreatic cell lineages (as described previously) and whether they are solid, cystic or intraductal (Bardeesy and DePinho, 2002; Hezel et al., 2006; Klimstra et al., 2009). As stated, there are multiple tumour types, however, pancreatic ductal adenocarcinoma (PDAC) is by far the most common pancreatic neoplasm and accounts for over 80% of cases (Ghaneh et al., 2007; Hezel et al., 2006). Because PDAC is the most commonly occurring cancer of the pancreas, the term PDAC and pancreatic cancer are often used synonymously but it should be noted that there are a diverse range of malignant neoplasms with different characteristics and biological/clinical outcomes (Reid et al., 2013). These are outlined in **table 1.2**.

Pancreatic neoplasm	Histological features	Genetic alterations	%
Ductal adenocarcinoma	Ductal morphology and desmoplasia	*KRAS, p16^{INK4a}, TP53, SMAD4*	85
Variants of PDAC:			
a. Medullary carcinoma	Poorly differentiated with intratumoral lymphocytes	*hMLH1, hMSH2*	
b. Colloid (mucinous noncystic) carcinoma	Mucin pools	*MUC2* overexpression	1-3
Intraductal papillary mucinous neoplasm	Papillary formation of mucin producing cells with duct dilation	*TP53, KRAS*	3-5
Pancreatic endocrine neoplasm	Hormone production	*MEN1*	3-4
Serous cystadenoma	Multilocular cysts and a glycogen-rich epithelium	*VHL*	1-2
Mucinous cystic neoplasm	Mucin producing cells with ovarian type stroma	*KRAS*	1-2
Solid pseudopapillary neoplasm	“Pseudo” papillae with both solid and cystic areas and hyaline globules	*APC/β-catenin* and CD10 expression	1-2
Acinar cell carcinoma	Zymogen granules	*APC/β-catenin*	1-2
Pancreatoblastoma	Squamoid nests and multilineage differentiation	*APC/β-catenin*	<1

Table 1.2: Classification of pancreatic neoplasms. Table describes an abridged classification of pancreatic neoplasms showing histological features and common genetic alterations. Table was adapted from: (Hezel et al., 2006; Klimstra et al., 2009).

Having stated that the majority of pancreatic neoplasms are ductal in origin, the actual cellular origin is still an ambiguous subject. There is a significant amount of diversity in the turnover of tissue in the pancreas, with a range of cells thought to contribute to homeostatic tissue turnover and/or regeneration of the pancreas. These include mature epithelial cells, stem cells and bone marrow cells (Kong et al., 2011). Due to the histologic appearance of PDAC, with a predominantly epithelial cell morphology being observed and dysplasia occurring in precancerous ductal lesions, a ductal origin has long been suggested (Hernandez-Munoz et al., 2008; Rooman and Real, 2012). However, due to the variety of cell types that contribute to normal tissue turnover, and with the development of genetic mouse models, it has been suggested that PDAC could arise from alternative cellular origins (Stanger and Dor, 2006).

With the development of conditionally activatable KRAS alleles in a mouse model, which perfectly mimics the point mutations leading to PDAC in the human pancreas (Aguirre et al., 2003; Hingorani et al., 2003), further insight has been provided into the cellular origins of PDAC. Carcinogens adversely impact various cell types in the pancreas, making lineage fidelity difficult to preserve (Habbe et al., 2008; Murtaugh and Leach, 2007) and, surprisingly, expression of oncogenic K-ras into ductal cell lines has failed to produce PDAC or its pre-cursor lesions (see **section 1.1.5**) (Brembeck et al., 2003). Numerous studies have implicated acinar origin due to the frequent observation whereby acinar cells expressing mutant K-ras transdifferentiate to produce a ductal cell phenotype in a process called acinar to ductal

metaplasia (ADM), which has been shown in both humans and mouse models (Carriere et al., 2007; De La et al., 2008; Guerra et al., 2007; Habbe et al., 2008; Morris et al., 2010a; Shi et al., 2013; Stanger and Dor, 2006). For example, it has been demonstrated that ectopic induction of the ductal gene *Sox9* when expressed with oncogenic K-ras induced the formation of PDAC precursors with ductal features (Kopp et al., 2012). In this same study, similar results were not observed when using either centroacinar or ductal cells.

Alternatively, studies into other possible cellular origins have also been published. It has been suggested that deregulated phosphatidylinositol 3-kinase (PI3K) signalling in centroacinar cells can also contribute to the development of PDAC *in vivo* (Stanger et al., 2005). In addition, centroacinar cells have been hypothesised to be pancreatic progenitor cells, contributing to maintenance of tissue homeostasis and exhibiting expansion in response to injury (Rovira et al., 2010). However, despite this study demonstrating the ability of centroacinar cells to differentiate, there is still little research to establish whether they play a definitive role in PDAC development (Rooman and Real, 2012). In addition, whether islet cells could be the cellular origins of PDAC has also been investigated. This hypothesis was based on results demonstrating that islet cells were capable of transdifferentiation, act as facultative stem cells and redifferentiate into cells with stem cell characteristics (Bardeesy and DePinho, 2002; Hennig et al., 2004). It has also been shown that after transplantation of islet cells into mice, pancreatic cancer with a PDAC-like phenotype developed in response to treatment with

the carcinogen nitrosamine *N*-nitrosobis-(2-oxopropyl)amin (BOP) (El-Ghamari et al., 2011). Furthermore, a recent study compared the localisation of the embryonic stem cell marker, Nanog, and the adult intestine stem cell marker, leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), in normal and pancreatic cancer samples. This revealed a novel colocalisation of the two proteins in beta cells within the islets and also in ductal cancer cells (Amsterdam et al., 2013). The authors suggest that the beta cells expressing these markers are the initiating cells of PDAC after migration and de-differentiation. There is also the question as to whether there is a population of stem cells within the pancreas that could give rise to PDAC. These cancer stem cells (CSCs) have a high proliferative capacity, have the potential to self renew and are pluripotent, with the ability to produce differentiated progeny (Bao et al., 2010; Li et al., 2007; Matsuda et al., 2012). These CSCs were shown to form tumours in immunocompromised mice and several CSC-specific marker have been reported, including CD133, CD24, CD44, CXCR4, EpCAM, ABCG2, c-Met, ALDH-1 and nestin, with upregulation of the Notch signalling pathway also being apparent. (Abel et al., 2014; Amsterdam et al., 2013; Li et al., 2011a; Matsuda et al., 2012; Rooman and Real, 2012). However, their use is controversial due the expression of these markers in some differentiated cell types of the adult pancreas (Rooman and Real, 2012).

1.1.5 Pathophysiology of PDAC

Over the last decade, knowledge of PDAC precursor lesions has grown and the general consensus is that there is a step-wise progression from these

lesions to invasive cancer (Distler et al., 2014). Three histologically different pancreatic tumour precursor lesions have been described: mucinous cystic neoplasms (MCN), intraductal papillary mucinous neoplasms (IPMN) and pancreatic intraepithelial neoplasias (PanIN) (Chu et al., 2007; Ghaneh et al., 2007; Hernandez-Munoz et al., 2008). Early detection and treatment of these precursor lesions could help prevent the subsequent development into invasive PDAC. PanINs are the most comprehensively studied and are the most commonly occurring precursor to PDAC, however, all of these lesions exhibit a characteristic step-wise progression towards PDAC and share a number of key features (Chu et al., 2007).

IPMNs are characterised by columnar, mucin-producing epithelium (Singh and Maitra, 2007), which typically arise within the main pancreatic duct and/or the major ductal branches (Werner et al., 2012). Approximately 70% of IPMNs are located in the head of the pancreas as opposed to the body or tail (Scarlett et al., 2011) and are subdivided into main duct (MD-IPMN) and branch duct (BD-IPMN) according to their specific site of origin. Where main and branch ducts are involved, mixed-duct or combined-duct terminology is applied (Distler et al., 2014; Fritz et al., 2012; Nagai et al., 2008). IPMNs are further subclassified depending on the degree of dysplasia into low-grade, intermediate-grade, high-grade and IPMN with associated invasive carcinoma (Distler et al., 2014) as well as by their histological appearance into intestinal, pancreatobiliary, gastric and oncocytic types (Grutzmann et al., 2010). Two types of invasive pancreatic cancers typically develop from IPMNs, tubular and colloid mucinous noncystic carcinomas (Grutzmann et

al., 2010; Xiao, 2012; Yopp and Allen, 2010). Common genetic aberrations within IPMNs include mutations in KRAS, CDKN2a, STK11 and a minor fraction of cases exhibit alterations to TP53 and SMAD4 (Grutzmann et al., 2010; Scarlett et al., 2011; Shi and Hruban, 2012). It has also been noted that in over 96% of IPMNs there is a mutation in either GNAS or KRAS, with a mutation in both genes being observed in half. In addition, GNAS mutations occurred more frequently in the intestinal subtype, whereas KRAS mutations were more prevalent in the pancreatobiliary subtype of IPMN (Furukawa et al., 2011; Kanda et al., 2013; Wu et al., 2011b).

The most infrequent precursor lesions of pancreatic cancer are MCNs and the majority are located within the pancreatic body and tail (Distler et al., 2014). They are typically described as neoplasms composed of mucin-producing epithelial cells and have an ovarian-type of stroma, with the majority being found in women (Scarlett et al., 2011; Testini et al., 2010). MCNs are cystic lesions, capable of growing very large, with a fibrous pseudocapsule, often with calcifications. Unlike IPMNs, MCNs rarely involve the ductal system (Zamboni et al., 1999). They are classified by the degree of dysplasia observed, which correlates with patient prognosis (Fernandez-del Castillo and Warshaw, 1995). KRAS mutations have been observed in early MCN lesions, which increase with advancing dysplasia. In more advanced MCNs, mutations in TP53, p16, SMAD4 have also been reported (Baker et al., 2012; Jimenez et al., 1999; Matthaei et al., 2011; Scarlett et al., 2011; Singh and Maitra, 2007).

PanINs are the most widely studied precursor of pancreatic cancer and were originally called by multiple terminologies, such as flat hyperplasia and ductal papillary hyperplasia (Scarlett et al., 2011), but the PanIN classification is now the accepted system (Hruban et al., 2001; Hruban et al., 2004). PanIN lesions are categorised into early and late lesions, progressing from PanIN-1A and PanIN-1B, which are low grade lesions to PanIN-2 and PanIN-3 lesions, which are intermediate and high-grade lesions, respectively (Distler et al., 2014; Ghaneh et al., 2007). These are described in **table 1.3**.

Lesion	Description
PanIN-1A	Develops from normal, flat epithelium and is composed of tall, columnar cells with a small amount of overcrowding. Nuclei are basally located and appear small and round/oval. There is also supranuclear mucin present.
PanIN-1B	These are very similar to PanIN-1A lesions, but columnar cells become increasingly crowded. Cells can also develop papillary projections, which often contain a stromal core.
PanIN-2	Architecturally these lesions are comparable to PanIN-1B lesions, commonly with papillary/micropapillary architecture. However, they demonstrate a moderate degree of architectural and nuclear atypia. These abnormalities include some loss of polarity, nuclear crowding and enlargement, nuclear stratification and hyperchromatism.
PanIN-3	These lesions demonstrate severe cytological and architectural atypia. Cribriform structures and luminal necrosis is often observed as small clusters of cells budding off into the gland lumen. There is a complete loss of nuclear polarity. They are also known as carcinoma *in situ* as lesions resemble carcinoma at the cytonuclear level. There is however, no invasion through the basement membrane.

Table 1.3: Description of PanIN lesions. PanIN lesions are precursors of pancreatic cancer and form a recognised tumour progression model with increasing neoplastic potential. Table adapted from: (Biankin et al., 2004; Hruban and Fukushima, 2007; Scarlett et al., 2011)

PanINs are microscopic lesions, usually less that 5mm, which form in the smaller, intralobular pancreatic ducts (Distler et al., 2014; Hruban and Fukushima, 2007; Hruban et al., 2004; Scarlett et al., 2011). Pancreatic cancer is thought to develop from low-grade to high-grade PanIN lesions, then PDAC. The increasingly disorganised cellular architecture and nuclear atypia is accompanied by a series of genetic changes (Distler et al., 2014) and is summarised in **figure 1.2**. There are a number of genetic aberrations that occur during the development and progression of PanIN lesions. Early events that take place include mutations in KRAS (De La et al., 2008), telomere shortening (Distler et al., 2014) and overexpression of $p21^{WAF1/CIP1}$ (Biankin et al., 2004), which is an important regulator of the cell-cycle machinery and its increased expression correlates with PanIN progression (Biankin et al., 2004; Scarlett et al., 2011). These early, activating mutations of KRAS are observed in over 90% of PDACs and are thought to be the driving force behind PDAC formation (Hingorani et al., 2003; Hruban et al., 1993). As stated, telomere shortening occurs early within PanIN lesion progression, this ultimately leads to abnormal fusion of chromosomes, affects cell division and increases genomic instability (Koorstra et al., 2008; Maitra et al., 2006; van Heek et al., 2002). Telomere shortening is thought to be one of the major causes for the gain of oncogenes and other genetic abnormalities include inactivation of several tumour suppressor genes (Haugk, 2010). These include inactivation of p16/CDKN2A occurring in early PanIN lesions and TP53 and SMAD4/DPC4 inactivation occurring later (Hruban et al., 2004; Koorstra et al., 2008). It has also been shown that aberrant CpG island hypermethylation begins in early PanIN lesions and this progressively

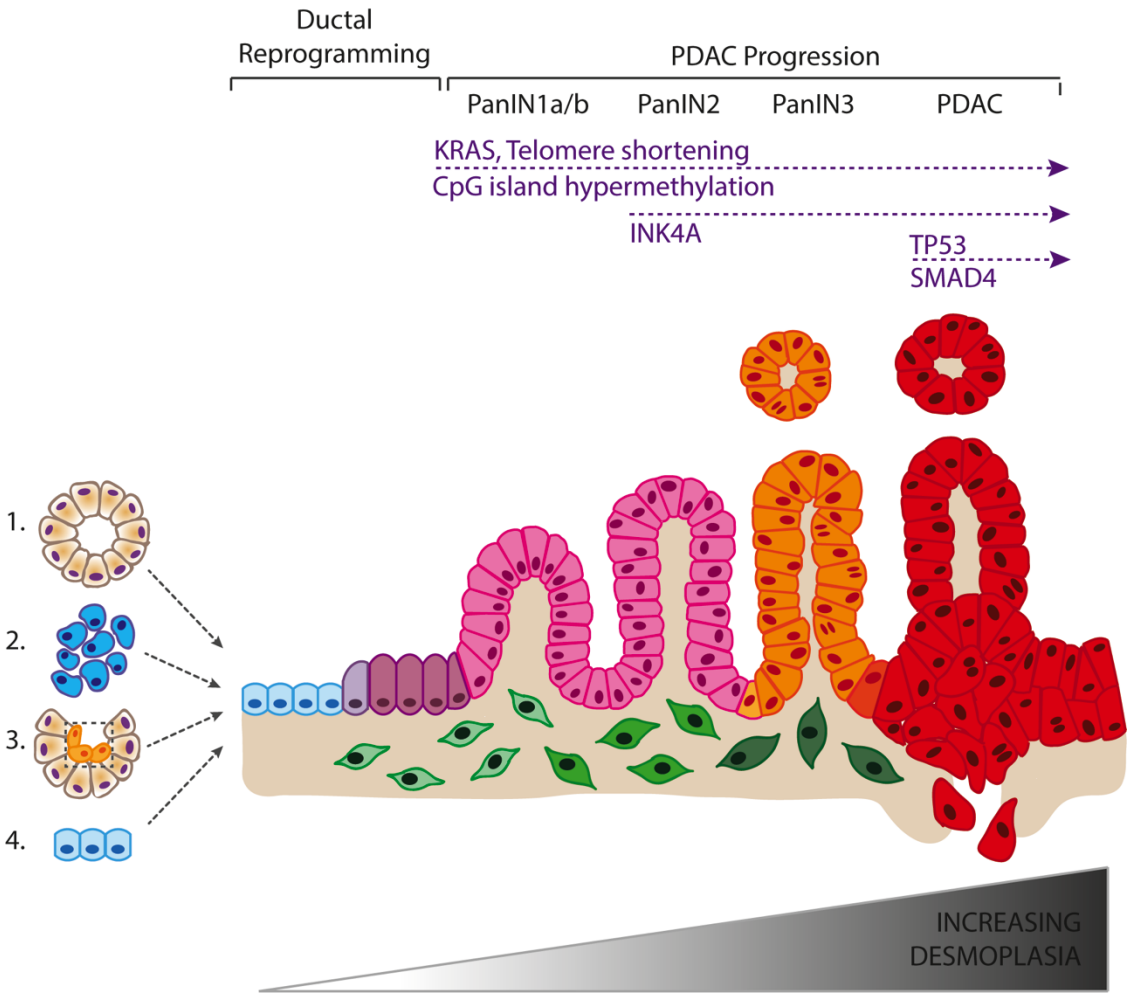


Figure 1.2: PanIN progression. Diagram demonstrates the different theorised cellular origins of PDAC including 1) acinar cells, 2) islet cells, 3) centroacinar cells and 4) duct cells. Activating KRAS mutations in pancreatic cells is able to initiate the development of PDAC. It is thought that this develops through a series of stages; progressing from PanIN-1A where the normal epithelium becomes tall and columnar to PanIN-3, which is also known as carcinoma *in situ*. With each progressive stage, subsequent mutations occur and accumulate including mutations in INK4A, TP53 and SMAD4. These mutations, in combination with telomere shortening and CpG island hypermethylation leads to increased genomic instability and atypical cellular architecture. In addition, the progression through these stages is accompanied by increasing desmoplasia, a hallmark of pancreatic cancer, whereby cancer-associated fibroblasts and activated pancreatic stellate cells contribute to an intense stromal reaction, contributing to PDAC development. Figure is adapted from: (Bardeesy and DePinho, 2002; Distler et al., 2014; Hernandez-Munoz et al., 2008; Morris et al., 2010b; Scarlett et al., 2011)

increases during PanIN progression (Sato et al., 2008). Furthermore, CyclinD1 has been shown to be increasingly overexpression with progressing PanIN lesion development, the tumour marker, prostate stem cell antigen (PSCA) is expressed in all PanINs, but not normal duct epithelia (Argani et al., 2001; Haugk, 2010; Maitra et al., 2003) and matrix metalloproteinase (MMP)-7 is expressed in PanIN lesions, which suggests the onset of its expression occurs early in PDAC development (Crawford et al., 2002). Differential MUC expression is observed in different PanIN lesions (Haugk, 2010), cathepsin E has also more recently been identified as a novel biomarker of PanIN/PDAC formation (Cruz-Monserrate et al., 2012). It is also thought that coordinated overactivation of both Hedgehog and Ras signalling pathways contributes heavily to promotion of PanIN lesion formation in the early stages of PDAC (Pasca di Magliano et al., 2006). However, there is still much that isn't fully understood about all of these PDAC precursor lesions and although they all follow a multi-step progression towards invasive PDAC, each lesion is characterised by its own distinct clinicopathologic and genetic alterations (Distler et al., 2014; Hernandez-Munoz et al., 2008; Scarlett et al., 2011).

1.1.6 Stromal contribution to PDAC

As described in **figure 1.2**, the desmoplastic reaction (DR) is one of the hallmarks of pancreatic cancer and involves the proliferation of fibrotic tissue. This DR is composed of extracellular matrix (ECM) proteins - predominantly type I collagen and fibronectin - fibroblasts, stellate cells, immune and inflammatory cells, lipocytes, endothelial cells and neurons (Bardeesy and

DePinho, 2002; Pandol et al., 2009; Rasheed et al., 2012). In addition to the characteristic dense bundles of collagen, there is a loss of basement membrane integrity and invasion of malignant cells into the interstitial matrix (Mahadevan and Von Hoff, 2007). The epithelial and stromal compartments communicate and interact, which is known to enhance the aggressive nature of PDAC (Chu et al., 2007; Erkan et al., 2012b; Merika et al., 2012; Whatcott et al., 2012). Furthermore, it is this desmoplastic reaction and the tumour microenvironment that are seen as the major contributors of chemoresistance in PDAC (Whatcott et al., 2012), due to deposition of ECM components leading to reduced elasticity of tumour tissue, increased tumour interstitial fluid pressure and subsequent decreased rate of perfusion of chemotherapeutic agents, ultimately reducing drug efficacy (Heldin et al., 2004).

There are numerous different cell types contributing to desmoplasia; of particular interest though, are the reciprocal interactions between tumour and stromal cells, which contributes to tumour progression and aggressiveness (Erkan et al., 2012b; Tang et al., 2013). In the normal pancreas, pancreatic stellate cells (PSCs) have a low proliferative rate (Beacham and Cukierman, 2005) and can be identified based on their expression of desmin and glial fibrillary acidic protein (GFAP) (Rasheed et al., 2012). It is already known that PSCs contribute to fibrosis, associated with chronic pancreatitis (Masamune et al., 2009) and that they become activated in response to oxidative stress and through the secretion of cytokines and growth factors from cancer cells (Erkan et al., 2012b). Upon activation, PSCs acquire

myofibroblastic characteristics such as α-smooth muscle actin (SMA) expression (Apte et al., 2004; Apte et al., 2013; Rasheed et al., 2012) and have an increased proliferative capacity. It has been shown that these activated PSCs secrete interleukin-6 (IL-6), which activates signal transducer and activator of transcription 3 (STAT3) and promotes anchorage-independent growth and invasiveness in PDAC cells (Guo et al., 2014a) and also prevent an effective antitumour response of CD8(+) T cells through secretion of CXCL12, which appears to act as a chemoattractant and therefore reduces T cell migration to juxtatumoural compartments (Ene-Obong et al., 2013). Furthermore, in direct coculture PSCs promote radioprotection of pancreatic cancer cells and stimulate proliferation (Mantoni et al., 2011).

Further interaction between the tumour and stromal compartments occurs through the release of several growth factors including transforming growth factor-beta (TGF-β), platelet derived growth factor (PDGF), fibroblast growth factor (FGF)-2, connective tissue growth factor (CTGF) and Sonic Hedgehog (SHH) signalling (Bailey et al., 2008; Damhofer et al., 2013; Gore and Korc, 2014; Lohr et al., 2001; Mahadevan and Von Hoff, 2007; Whatcott et al., 2012). A recent study using a well-defined mouse model of PDAC demonstrated that SHH-deficient tumours had a reduction in tumour-associated stroma, as would be predicted. Interestingly, however, these tumours were actually more aggressive with increased vascularity and proliferative capacity and concluded that SHH-driven stroma formation could, in part, act to restrain tumour growth (Rhim et al., 2014). However, the cross-

talk between tumour and stromal compartments and the activation of autocrine and paracrine oncogenic signalling pathways is known to contribute heavily in the desmoplastic reaction, which is a hallmark of PDAC. A better understanding of this cross talk will help identify possible therapeutic strategies.

1.2 Molecular genetics of PDAC

1.2.1 Signalling overview

Pancreatic cancer is characterised by rapid growth, desmoplasia, early invasion and resistance to chemotherapy (Hidalgo, 2010). This can be heralded to the accumulation of many genetic alterations/mutations that occur in an ordered sequence during the development and multistep progression of PDAC. These inherited and acquired (somatic) mutations ultimately impact on a number of signalling pathways; genomic analysis in a screen of 24 different PDAC samples revealed that there are, on average, 63 genetic alterations found within each sample. These are thought to impact on 12 main, overlapping, signalling pathways (Jones et al., 2008; Sakorafas et al., 2010), which emphasises the intense nature and complexity of the disease (Ottenhof et al., 2011). These pathways involve cellular functions; apoptosis, homophilic cell adhesion, invasion, DNA damage repair, G1/S phase cell cycle progression and different signalling cascades; Hedgehog, integrin, c-Jun N-terminal kinase (JNK), K-ras, TGF-β, Wnt/Notch and small GTPase signalling (other than K-ras) (Jones et al., 2008; Ottenhof et al., 2011). The mutations in these pathways accumulate with increasing genomic instability, which occurs primarily due to telomere shortening (van Heek et al., 2002). Although PDAC is associated with many mutations in cancer-associated genes (oncogenes, tumour-suppressor genes and genome-maintenance genes), genetic and molecular pathways (telomere shortening), upregulation and overexpression of growth factors and their associated receptors (EGFR, c-Met and TGF-β, for example), angiogenesis and altered developmental pathways (Hedgehog) (Sakorafas et al., 2010) for the

purposes of this review, the focus will be placed on ras signalling, hepatocyte growth factor (HGF) and their downstream pathways including c-Met, PI3K and AKT. However, the main genetic/molecular alterations involved in PDAC are summarised in **table 1.4**.

Function	Details	Key References
Apoptosis	BCL-2, BCL-X(L), MCL-1, caspase inhibitor (survivin) overexpression NF-κB and PI3K/AKT signalling Hyper-*O*-GlcNAcylation (maintains NF-κB signalling) CASP10, VCP, CAD, HIP1	(Arlt et al., 2013; Hamacher et al., 2008; Ma et al., 2013)
Adhesion	E-cadherin, α/β-catenin – reduced expression CDH1, CDH10, FAT, PCDH15, PCDH17	(Froeling et al., 2009; Joost et al., 2012)
Invasion	KRAS mutation drives expression of stable mutant of p53 SDC-2 invasion-associated gene in PDAC – cooperates with K-ras ADAM11, DPP6, MEP1A, PCSK6, APG4A, PRSS23	(De Oliveira et al., 2012; Morton et al., 2010; Weissmueller et al., 2014)
DNA damage repair	TP53 expression lost in 50-75% PDAC MLH1, MSH2, MSH6, PMS2 – mismatch repair genes (microsatellite instability) BRCA2 – familial and sporadic PDAC (interstrand cross-linking repair) ERCC4, ERCC6, EP300, RANBP2	(Maginn et al., 2014)
G1/S transition	CDKN2A/p16 (FBXW7, CHD1, APC2 – less frequent that CDKN2A) Sox2 – aberrantly expressed controls genes involved in transition	(Herreros-Villanueva et al., 2013)
JNK signalling	Loss of function mutations in activators of JNK, mitogen-activated protein kinase (MAPK) kinase 4 (MKK4) and MKK7 impairing JNK signalling Interacts with KRAS signalling TNF, ATF2, NFATC3	(Davies et al., 2014)

Integrin signalling	Mutant p53 drives mutations by promoting integrin recycling ITGA4, ITGA9, LAMA1, FN1, ILK	(Muller et al., 2009; Tuveson and Neoptolemos, 2012)
Small GTPase signalling	RalB – invadopodia formation Rio kinase 3 (RIOK3) – gene amplification – increases invasiveness through activation of rac AGHGEF7, CDC42BPA, DEPDC2, PLCB3	(Kimmelman et al., 2008; Neel et al., 2012)
K-ras signalling	KRAS, MAP2K4, RASGRP3 30% of early neoplasms and >90% PDAC Activates RAF/ERK, PI3K, RalGDS and NF-κB pathways	(Eser et al., 2013; Eser et al., 2014)
TGF-β signalling	SMAD4 (loss of heterozygosity (LOH) in 90% PDAC), TGFBR2, BMPR2, SMAD3 Additional inactivation of remaining allele in 50% PDAC Promotes proliferation and epithelial to mesenchymal transition (EMT)	(Ijichi et al., 2006; Javle et al., 2014)
Wnt/Notch signalling	Upregulation/Interacts with Hedgehog, KRAS and NF-κB signalling pathways MYC, WNT9A, GATA6, TCF4, MAP2, TSC2	(Froeling et al., 2011; Miyamoto et al., 2003; Pasca di Magliano et al., 2006; Tremblay et al., 2013)
Hedgehog signalling	Activated in 70% PDAC, essential for CSC viability, paracrine signalling impacts stromal cell activity Activated by galectin-1 (Gal1) overexpression TBX5, SOX3, LPR2, GLI1, BOC, CREBBP	(Martinez-Bosch et al., 2014; Nolan-Stevaux et al., 2009; Seton-Rogers, 2009; Yauch et al., 2008)

Table 1.4: Summary of molecular events involved in PDAC. The 12 main signalling pathways affected by genetic mutations in pancreatic cancer. Additional references: (Cowley et al., 2013; Hezel et al., 2006; Jones et al., 2008; Mihaljevic et al., 2010; Morris et al., 2010b; Ottenhof et al., 2011)

1.2.2 Ras

In humans, there are three RAS genes, which encode four distinct proteins, H-ras, N-ras, K-ras4A and K-ras4B; K-ras4A and K-ras4B are alternative splice variants of the KRAS gene (Bar-Sagi, 2001; Castellano and Santos, 2011; Lowy and Willumsen, 1993; Plowman et al., 2003; Pylayeva-Gupta et al., 2011). H-, N- and K-ras4A are each 189 amino acids in length while K-ras4B, which is found more abundantly than K-ras4A, is 188 amino acids; all are approximately 21 kilodaltons (kDa) (Bar-Sagi, 2001; Pylayeva-Gupta et al., 2011). The structure of all four ras isoforms is very similar and can be divided into three main regions (**figure 1.3**). First, the N-terminus, which is identical across the four isoforms, is 86 amino acids in length; with amino acids 32-40 constituting a ras effector-protein binding domain (Bar-Sagi, 2001), which allows interaction with downstream effector proteins and enables participation in a wide range of signal transduction cascades. The second mid-region is 80 amino acids and although not identical, is still highly homologous (Welman et al., 2000). The remaining section, starting at amino acid 165, shares no sequence homology between any of the isoforms, except for a conserved CAAX motif (C – cysteine, A – aliphatic amino acids, X – methionine or serine). This motif plays an important role in directing post-translational modifications including farnesylation, which affords ras affinity for cellular membranes. This association with the cytoplasmic surface of cellular membranes is a prerequisite of ras activation and signalling (Ahearn et al., 2012; Castellano and Santos, 2011).

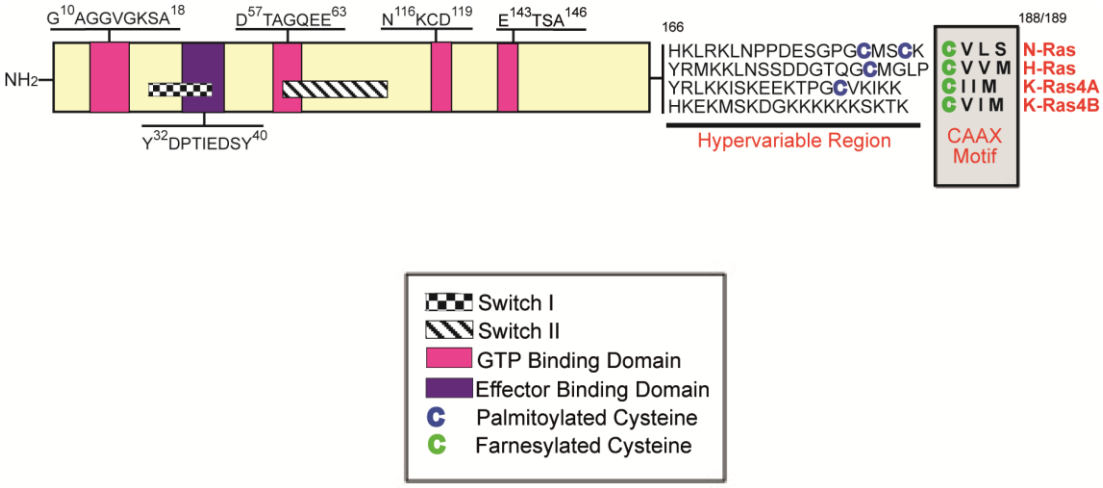


Figure 1.3: Structure of ras proteins. There are four ras isoforms, which are products of three RAS genes, with K-ras4A and K-ras4B being alternative splice variants of the KRAS gene. The N-terminus is highly conserved across all four isoforms and includes binding sites for GDP and GTP and downstream effector proteins. The C-terminus, from amino acid 165-189 (188 for K-ras4B) shares no sequences homology between the isoforms, with exception of a conserved CAAX motif. This plays an important role in directing post-translational modifications such as farnesylation and palmitolation.
Figure adapted from: (Castellano and Santos, 2011)

Under normal cellular conditions, ras is found in guanosine diphosphate (GDP)/inactive and guanosine triphosphate (GTP)/active forms. To become active, GDP must first dissociate; this is an intrinsically slow process, but is accelerated by guanine nucleotide exchange factors (GEFs). Once GDP has dissociated, GTP can bind in its place. Within this active conformation, ras GTPases are able to interact with various effector proteins to impact on different cellular processes including cell growth, proliferation, migration and apoptosis (Malumbres and Barbacid, 2003) and this signalling is summarised in **figure 1.4**. The hydrolysis of GTP to GDP, which is mediated through intrinsic GTPase activity, reverts ras back to an inactive state; this process is accelerated through the action of GTPase activating proteins (GAPs) (Biou and Cherfils, 2004; Colicelli, 2004; Vetter and Wittinghofer, 2001). Structural changes that occur within the overlapping motile switch I and switch II regions in response to guanine nucleotides also affect the interactions of ras with GAP and GEF regulators (Castellano and Santos, 2011). The switch regions are sensitive to the nature of the bound guanine nucleotide and change their structural conformation in response. This increases the affinity for the bound nucleotide and ensures the complex remains bound until the reaction is complete (Biou and Cherfils, 2004; Hall et al., 2002).

1.2.3 Oncogenic K-ras signalling in PDAC

The KRAS gene is frequently mutated in PDAC and has been found to be present in over 90% of patient tumour samples and occurring in even low-grade PanIN-1A lesions (Eser et al., 2014; Kanda et al., 2012; Morris et al., 2010b). As stated, under normal cellular conditions, K-ras cycles between an

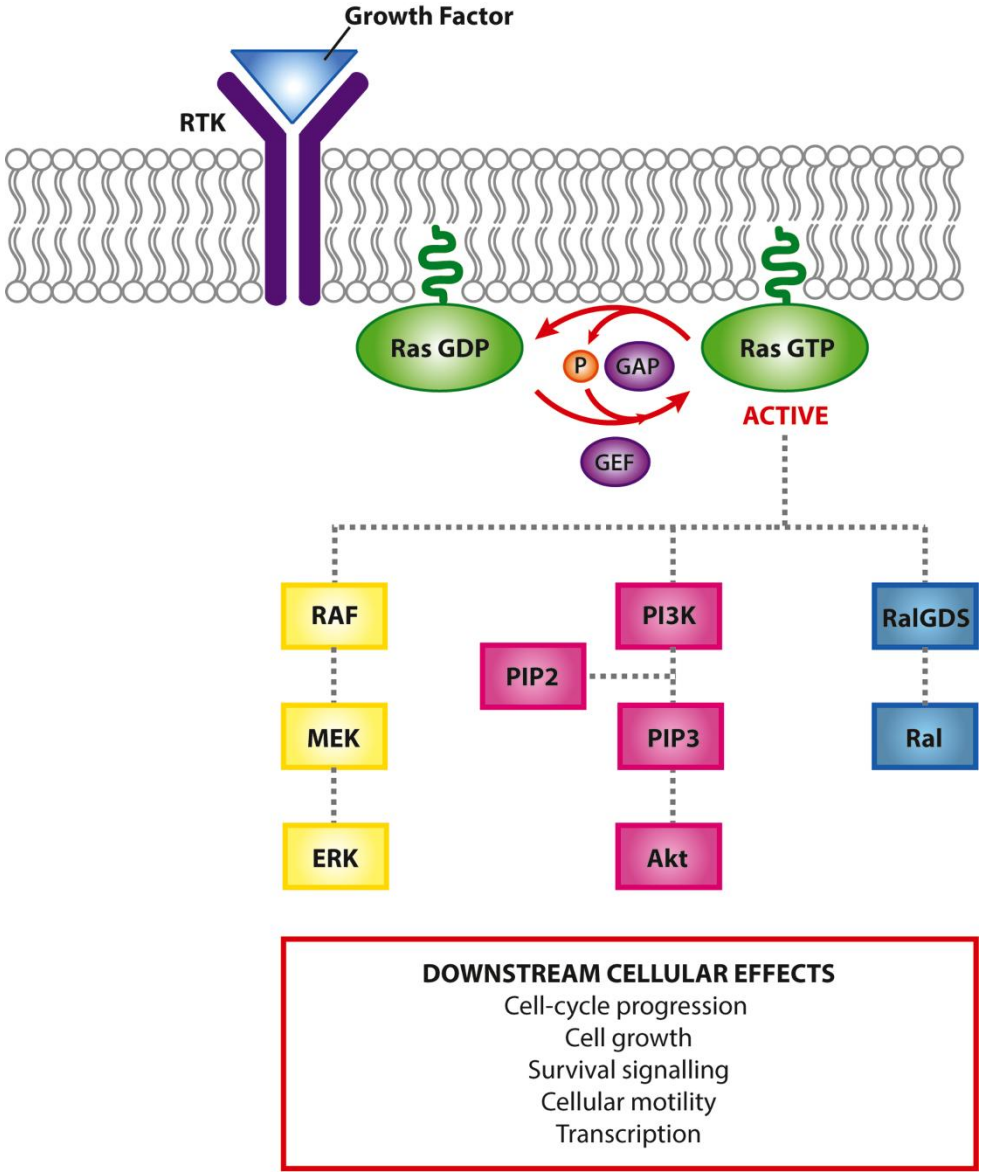


Figure 1.4: Ras signalling. Ras is found bound to GDP (inactive) and GTP (active). The transition between the two states is regulated via GEFs and GAPs. GAPs accelerate the intrinsic GTPase activity of ras, releasing a phosphate group and converting ras back to its inactive state. GEFs mediate the exchange of GDP for GTP, activating ras and allowing its interaction with a multitude of effector proteins to participate in signalling pathways. Mutations in K-ras occur frequently in PDAC leading to continual activation of these downstream signalling pathways. Depicted are the three major cascades implicated in PDAC progression: RAF/MEK/ERK, PI3K and RalGDS.
Figure adapted from: (Downward, 2003; Hezel et al., 2006; Hruban et al., 2008)

inactive (GDP-bound) and active (GTP-bound) form (Malumbres and Barbacid, 2003), however, activating mutations that occur within PDAC render it constitutively active (Hezel et al., 2006). The most common mutation is on codon 12 (Collins et al., 2012; Jones et al., 2008) from GGT to GAT or GTT and very rarely CGT, whereby glycine (G) is substituted with aspartic acid (D), valine (V) or arginine (R) (Collins and Pasca di Magliano, 2013; Eser et al., 2014; Hezel et al., 2006; Rachakonda et al., 2013). Glycine to aspartic acid (K-ras^{G12D}) substitutions are the most common (Eser et al., 2014; Pylayeva-Gupta et al., 2011). These mutations compromise the intrinsic GTPase activity of ras, meaning it is unable to hydrolise GTP to GDP; they can also block the interaction between K-ras and GAPs (Collins and Pasca di Magliano, 2013; Eser et al., 2014). This ultimately means that K-ras is locked in an active state and leads to persistent activation of downstream signalling pathways, many of which are key drivers of PDAC initiation and progression (Pylayeva-Gupta et al., 2011).

Although KRAS mutations are sufficient for the initiation of pre-cursor lesions, additional genetic aberrations are required for disease progression (Morris et al., 2010b) and K-ras driven genetically engineered mouse models (GEMM) of PDAC can be accelerated through introduction of inactivating mutations of the tumour suppressor genes CDKN2A, TP53 and/or DPC4/SMAD4 (Biankin et al., 2012; Eser et al., 2014). Indeed, sustained K-ras signalling is an essential requirement for maintenance of metastatic lesions, PDAC progression and growth; genomic analysis has revealed a fundamental role for K-ras in reprogramming tumour metabolism through selective activation of

biosynthetic pathways, which support tumour growth (Ying et al., 2012). Knowing that K-ras is crucial in PDAC progression, and with cell line studies suggesting an oncogenic KRAS addiction (Zimmermann et al., 2013), it makes K-ras an attractive therapeutic target. However, attempts in producing potent and direct inhibitors to target ras have not been successful and have lead to the perception that ras is undruggable (Bryant et al., 2014). However, there have been some advances, with on-going research showing that targeting ras may still hold promise. These studies have focused on selected compounds have been used to inhibit son of sevenless (SOS)-mediated nucleotide exchange, therefore inhibiting SOS-catalysed K-ras activation. Another strategy has implemented small-molecule inhibitors that target Ras-GTP directly, which blocks ras interactions with downstream effector proteins (Maurer et al., 2012; Shima et al., 2013; Sun et al., 2012). It is known that PDAC shows high dependency on K-ras and sustained K-ras signalling is a requirement for PDAC maintenance (Ying et al., 2012). Recently, several studies have demonstrated a link between K-ras and the transcriptional coactivator Yes-associated protein-1 (YAP1). It was shown that in a GEMM of PDAC, following K-ras suppression there was initial tumour regression. However, the majority of tumours relapsed and it was shown that activating mutations in YAP1 were able to compensate for K-ras suppression. It has also been noted that YAP1 can induce EMT and proliferation in PDAC independently of K-ras (Greten, 2014; Kapoor et al., 2014; Seton-Rogers, 2014; Shao et al., 2014; Zhang et al., 2014b). These studies therefore provide evidence for the ability of YAP1 to substitute K-ras signalling in oncogenic K-ras dependent PDAC. However, to date, research has focused

on downstream effector pathways of oncogenic K-ras in PDAC as more optimistic therapeutic targets to combat the disease.

1.2.4 Downstream effector pathways of oncogenic K-ras

Within PDAC, it is believed that there are three main effector pathways downstream of K-ras; these are the MAPK, PI3K and RalGEF pathway. Through the activation of these various signalling cascades, active ras is able to impact multiple cellular processes that are critical to PDAC progression including, but not limited to, proliferation and survival, as well as cell polarity and migration (Downward, 2003). These pathways are summarised in **figure 1.4**. MAPK signalling is present in both early and late stage PDAC, in both mouse models and human tumours. It involves the activation of Raf kinases, by K-ras, which then activate MEK1/2. This results in phosphorylation and activation of ERK1/2 and subsequently affects cell-cycle progression, transcription, cell migration etc (Collins and Pasca di Magliano, 2013; Downward, 2003; Hingorani et al., 2003). RalGEFs are required for ras-induced transformation and activate ras-like small GTPases such as ral-A or ral-B, which promote PDAC growth and metastasis. In addition activation of CDK5, downstream of K-ras and increases malignant progression, migration and invasion *in vitro* is linked to ras-ral signalling (Eggers et al., 2011; Eser et al., 2014; Feldmann et al., 2010; Lim et al., 2005; Lim et al., 2006). However, the main focus of this study is the PI3K pathway, which is what will now be covered in depth.

Among the different oncogenic K-ras activated effector pathways that are involved in PDAC, the PI3K pathway is a key mediator of ras-driven oncogenesis and is emerging as one of the most critical (Eser et al., 2013); it has been estimated that approximately 50% of cancers have deregulation of this pathway involved in their tumorigenesis (Ferro and Falasca, 2014; Yuan and Cantley, 2008). PI3Ks are lipid kinases of which there are multiple isoforms; these can be divided into three classes, which is based on structural features and substrate preferences. Class I PI3Ks comprise a p110 catalytic subunit and a regulatory subunit. Class II PI3Ks share approximately 45% homology with the catalytic subunit of class I PI3Ks but have a C-terminal region that mediate calcium and lipid binding. Class III PI3Ks are thought to regulate vesicle trafficking (Cantrell, 2001). PI3Ks phosphorylate the 3'-hydroxyl group on the inositol ring of phosphoinositides to generate secondary messengers such as phosphatidylinositol-3,4,5-triphosphate (PIP_3), using phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2) as a substrate. PIP_3 recruits and coordinates downstream effectors, such as Akt, also known as protein kinase B (PKB), which then stimulates various effector pathways (Cantrell, 2001; Franke, 2008; Vanhaesebroeck et al., 2010; Yuan and Cantley, 2008). Class I PI3Ks are the best understood, which are further subdivided into Class IA (α, β and δ), which bind the p85 type of regulatory subunit and Class IB (γ), which binds either the p101 or p87 regulatory subunit (Vanhaesebroeck et al., 2010). It is the class IA PI3Ks, which are more heavily implicated in cancer (Yuan and Cantley, 2008; Zhao and Vogt, 2008), however, more recently it has been hypothesised that

p110γ overexpression could be a key player in PDAC progression (Edling et al., 2010).

Ras interacts with the P110 subunit via a RAS binding domain (RBD) and this is independent of p85 (Castellano et al., 2013; Gupta et al., 2007; Organ and Tsao, 2011). Upon activation, PI3Ks catalyse the phosphorylation of PIPs, which promotes recruitment of downstream effectors such as 3-phosphoinositide-dependent protein kinase-1 (PDK1) and Akt (Ferro and Falasca, 2014). The activity of PI3K is negatively regulated by the tumour suppressor phosphate and tensin homolog (PTEN) (Georgescu, 2010). Although mutations in PTEN are rare in PDAC, the disease is associated with a loss of PTEN function and this, in association with constitutively active K-ras leads to sustained PI3K signalling (Hill et al., 2010). In addition, PI3K signalling in the tumour microenvironment has been shown to significantly enhance PDAC progression (Ferro and Falasca, 2014) and activation of this pathway also increases NFκB signalling to promote tumour-enhancing changes to the tumour microenvironment (Ying et al., 2011).

As stated, PI3K signalling leads to the activation of Akt, which is a known indicator of aggressiveness in PDAC (Kennedy et al., 2011a; Kennedy et al., 2011b; Yamamoto et al., 2004) and correlates with outcome (Baer et al., 2013; Parsons et al., 2010; Schlieman et al., 2003). Akt is a member of the 'AGC' superfamily of protein kinases, of which there are 80 members; there are three isoforms of Akt (α, β, γ; Akt 1,2,3), which have differential expression patterns, with recent data suggesting they may have over-lapping

and distinct roles in cancer (Engelman, 2009; Franke, 2008; Scheid and Woodgett, 2003). Akt is activated by PIP_3, through PIP_3 binding via the pleckstrin homology (PH) domain, which causes a conformational change, unmasking the kinase domain and allowing phosphorylation to occur (Georgescu, 2010). Phosphorylation at two sites is required for full activation of Akt (Higuchi et al., 2008); PDK1 phosphorylates Thr308 in the activation loop and the mammalian target of rapamycin complex-2 (mTORC2) phosphorylates Ser473 in the C-terminal hydrophobic motif (Case et al., 2011; Georgescu, 2010; Higuchi et al., 2008; Moore et al., 2011; Sarbassov et al., 2005; Tato et al., 2011). There are two classes of phosphatases that are known to dephosphorylate and therefore inactivate Akt, protein phosphatase 2A (PP2A) and PH domain leucine-rich repeat protein phosphatases (PHLPP) 1 and 2 (Georgescu, 2010).

Activation or overexpression of Akt1 has been observed in human pancreatic tumour samples and Akt2 is also thought to be amplified or overexpressed in a small percentage of PDACs (Asano et al., 2004; Michl and Downward, 2005; Ruggeri et al., 1998; Semba et al., 2003). Activated Akt is known to promote cell survival, which it does through antagonisation of key apoptotic machinery components such as Bad (Datta et al., 1997; Fang et al., 1999), caspase-9 (Zhou et al., 2000) and forkhead transcription factor family members (Brunet et al., 1999). Within PDAC, Akt signalling has been shown to play heavily in chemoresistance through crosstalk with the chromatin modulator, BRG1 (Liu et al., 2014) and through regulation by the multi-functional cell membrane protein, RLIP76 (Leake et al., 2012). Furthermore,

pharmacological inhibition of components of the PI3K signalling module, including PI3K, PDK1 or Akt have been shown to inhibit PDAC cell proliferation *in vitro* (Baer et al., 2013) and genetic inactivation of PDK1 blocks oncogenic K-ras induced PDAC formation *in vivo* (Eser et al., 2013). However, although typically the PI3K/AKT pathway has been considered primarily to be responsible for survival signalling and proliferation, there is accumulating evidence to suggest that Akt signalling contributes to cellular motility (Kim et al., 2001; Xue and Hemmings, 2013; Xue et al., 2012). Akt has been shown to induce tumour metastasis through Twist1 phosphorylation-driven EMT and crosstalk between TGF-β and PI3K signalling (Xue et al., 2012) and activated Akt was found to promote phosphorylation/activation of components important cell migration including actin filaments (Ho et al., 2011). Furthermore, phosphorylation of Akt was required for motility of lung endothelial cells downstream of HGF (Usatyuk et al., 2014). It has also recently been reported that the Wnt family member, Wnt5A, promotes phosphorylation of Akt at Ser473 downstream of PI3K to promote osteosarcoma cell migration (Zhang et al., 2014a). However, although a role for Akt in cell migration, including in metastatic cancer cells, has been documented, whether Akt contributes pancreatic cancer cell migration has yet to be fully established.

1.2.5 HGF/c-Met signalling in PDAC

It is known that the PI3K pathway can be activated downstream of Ras. However, PI3K is also activated through association with receptors such as c-Met. The c-Met proto-oncogene codes for the c-Met protein, which is a cell

surface receptor tyrosine kinase (RTK). It is found expressed in a range of epithelial cells including those of the prostate, liver and pancreas both during development and throughout adulthood (Organ and Tsao, 2011). Post-translational, proteolytic processing produces a single-pass, disulphide-linked α/β heterodimer (Comoglio et al., 2008; Organ and Tsao, 2011). c-Met acts as a high affinity receptor for HGF, which is also known as scatter factor (Naldini et al., 1991; Weidner et al., 1991). Upon binding, HGF induces c-Met receptor dimerisation and acts as a pleiotropic factor and cytokine to initiate a phosphorylation cascade. Signalling through this receptor is key to a number of important biological and cellular processes, including development, wound healing and morphogenesis and promotes proliferation, cell survival, motility and cell scattering (Blumenschein et al., 2012; Boros and Miller, 1995; Brinkmann et al., 1995; Maulik et al., 2002; Zhu et al., 1994). HGF/c-Met signalling is known to play an important role in embryogenesis and wound repair through induction of cell scattering involving the dissolution of cadherin-based cell junctions and increased cellular motility (Chmielowiec et al., 2007).

HGF/c-Met signalling has been shown to play a significant role in pancreatic tumorigenesis too, acting upstream of several signalling pathways including PI3K and MAPK (Maehara et al., 2001; Patel et al., 2014; Tan and Yang, 2010). Under physiological conditions, both c-Met and HGF are expressed at low levels in acinar and stromal cells of the pancreas (Di Renzo et al., 1995). In many cancers, c-Met is mutated, but this is rare in PDAC; more often there is a marked increase in c-Met expression observed in PDAC tumour samples

(Di Renzo et al., 1995; Ebert et al., 1994; Furukawa et al., 1995; Yu et al., 2006), which correlates with poor prognosis (Zhu et al., 2011). It is also known that there are increased levels of circulating HGF in pancreatic cancer patients (Kemik et al., 2009).

The regulatory p85 subunit of PI3K serves three main roles: stabilisation, inactivation and recruitment (Vanhaesebroeck et al., 2010). To initiate PI3K signalling, in response to growth factor stimulation, the p85 subunit associates with the c-Met receptor. This interaction can either be direct, whereby the p85 subunit associates with phosphorylated tyrosine residues via its SH2 domain, or indirectly via the scaffold protein Gab1 (Engelman, 2009; Organ and Tsao, 2011; Rocchi et al., 1998; Yu et al., 2001). After this association of the SH2 domain on p85 and the phosphorylated tyrosine residues takes place, p85-mediated inhibition of the catalytic P110 subunit is relieved enabling p110 to transfer phosphate groups to initiate downstream signalling after being brought into contact with their lipid substrates at the cell membrane (Vanhaesebroeck et al., 2010). This is described in **section 1.2.4** and summarised in **figure 1.4**. In addition, c-Met mediated invasiveness and HGF-induced scattering have been shown to be enhanced through the bioactive peptide Neuromedin U (NmU), which is also found overexpressed in PDAC samples (Ketterer et al., 2009). Neuropilin-1 (Np-1) is also overexpressed in PDAC; Np-1 is a receptor for semaphorin 3A and vascular endothelial growth factor (VEGF), however, this has also shown to associate with c-Met in an HGF-induced manner to aid c-Met activation and promote PDAC cell invasiveness (Matsushita et al., 2007). Taken all together, these

data show the extreme complexity of the signalling pathways and cross talk involved in PDAC progression and the need to gain a better understanding of the roles they play in the various stages of the disease, including invasion and metastasis.

1.3 Cell migration and metastasis

1.3.1 Cell migration

Cell migration is central to a number of physiological processes occurring in many organisms. It is important in both embryonic development and for normal functioning within the adult organism (Kurosaka and Kashina, 2008; Ridley et al., 2003; Vicente-Manzanares et al., 2005). Cell migration plays a key role in gastrulation and in the formation of various tissues and organs of the embryo (Chuai et al., 2012; Solnica-Krezel and Sepich, 2012). Comparable cell migration occurs in adult organisms, contributing to the renewal of the skin and intestinal lining (Heath, 1996), morphogenesis (Juliano and Haskill, 1993), wound healing (Schneider et al., 2010) and immune responses (Ding et al., 2012; Madri and Graesser, 2000; Tomura et al., 2010). However, cell migration can also factor into various pathological processes including vascular disease and cancer (Chaffer and Weinberg, 2011; Lauffenburger and Horwitz, 1996; Vicente-Manzanares et al., 2005).

Migration of cells begins when the cells become polarised in response to external stimuli. This can take the form of various signals including chemical gradients of dissolved or surface-attached molecules (chemotaxis and hapotaxis) (Carter, 1965; Carter, 1967), light intensity (phototaxis) (Jekely, 2009; Saranak and Foster, 1997), electrostatic potential (galvanotaxis) (Erickson and Nuccitelli, 1984), gravitational potential (geotaxis) (Lowe, 1997) or matrix stiffness (durotaxis) (Lo et al., 2000).

There are a number of key steps involved in cell migration. Initially polarisation of the cell must occur in order for directional migration to commence, followed by a cyclical process combining adhesion to the ECM with protrusion, elongation, contraction and detachment of the rear of the cell (Huttenlocher and Horwitz, 2011; Lauffenburger and Horwitz, 1996; Ridley et al., 2003). Polarisation occurs in response to environmental stimuli and defines the leading and trailing edges of the cell. It is a complex process, requiring a high level of coordination between a number of key regulatory molecules. PI3K and its lipid products PIP_3 have been heavily implicated in control of cell polarity and migration. In response to environmental signals, it has been reported that PI3K becomes activated and causes the accumulation of PIP_3 at the leading edge of the cell. A concentration gradient of PIP_3 is reinforced due to the localisation of PTEN at the rear of the cell (Merlot and Firtel, 2003; Raftopoulou and Hall, 2004; Webb and Horwitz, 2003). There are a number of elegant studies in *Dicytostelium discoideum* demonstrating the requirement for a PIP_3 concentration gradient in cell migration (Funamoto et al., 2002; Iijima and Devreotes, 2002), however, more recent data has suggested that it may not be necessary for chemotactic cell migration in either *Dicytostelium discoideum* or neutrophils (Hoeller and Kay, 2007; Veltman et al., 2014), as previously described (Cai and Devreotes, 2011; Leslie et al., 2005). Despite this, a central role for PI3K/PTEN regulated PIP_3 gradient is still frequently cited.

Ras functions as an essential part of the cell migratory machinery, acting upstream of PI3K at the leading edge of the cell to initiate the formation of

this concentration gradient; a positive feedback loop then amplifies the concentration of PIP_3 to promote actin polymerisation (Sasaki and Firtel, 2005). This gradient leads to the activation of Rho GTPases such as Rho, Rac and cell division control protein-42 (Cdc42), which are known to play a central role in controlling the cellular responses required for cell migration. It has been reported that Rho promotes actin/myosin contraction in both the cell body and at the rear of the cell, Rac induces the formation of membrane protrusion at the leading edge through regulation of actin polymerisation and integrin adhesion complexes and Cdc42 mediates the direction of cell movement (Parri and Chiarugi, 2010; Raftopoulou and Hall, 2004; Ridley, 2001). Rac and Cdc42 target the Wiskott-Aldrich syndrome protein/WASP family verprolin homologous protein (WASP/WAVE) family of proteins; these in turn activate the actin related protein 2/3 (Arp2/3) complex (Friedl and Wolf, 2010; Ridley et al., 2003; Webb and Horwitz, 2003). This cascade of events subsequently leads to reorganisation and polymerisation of the actin cytoskeleton, which is needed to cause the cell to change shape and generate the forces required to drive cell migration (Hall, 1998). Cell migration requires the protrusion of cellular processes such as lamellopodium, filopodium, pseudopodia or invadopodia; although these structures are diverse in morphology, they all contain filamentous actin.

Actin is the most abundant protein found within all eukaryotic cells. It is found in two forms: monomeric, globular G-actin; and polymeric, filamentous F-actin, which forms from the polymerisation of G-actin into double helical filaments (Dominguez and Holmes, 2011; Holmes, 2009; Oda et al., 2009), a

reaction that is catalysed by ATP hydrolysis (Pollard and Borisy, 2003). Nucleation, whereby three G-actin monomers must associate to create a filament nucleus, is the process by which new actin filaments are created; this is the rate limiting step in actin polymerisation (Krause and Gautreau, 2014). Each actin monomer is orientated in the same direction, resulting in a polarised structure, with discernibly different ends. This is due to association of myosin, which creates an arrowhead pattern. The ends are defined, based on this pattern, as barbed (plus) or pointed (minus) (Pollard and Borisy, 2003). The nucleation of G-actin monomers occurs more readily at the barbed end of the actin filaments, which are orientated strongly with respect to the cells surface with barbed ends outwards and this rapid polymerisation pushes the membrane forward (Pollard and Borisy, 2003; Small et al., 1978), which is facilitated by a decrease in membrane tension (Raucher and Sheetz, 2000). In lamellipodia, Cdc42, Rac and/or WASP/WAVE proteins activate the Arp2/3 complex and cause nucleation of actin into a branching network, providing lamellipodia with their characteristic flat and broad morphology. Behind the dynamic lamellipodia is a region called the lamella. This is much more stable and couples the actin network to myosin II-mediated contractility (Ponti et al., 2004). In contrast, filopodia can be recognised by fine, finger-like projections, which are formed through promotion of linear actin polymerisation by the action of Cdc42 via formins, vasodilator-stimulated phosphoprotein (VASP) and fascin (Faix and Rottner, 2006; Jaffe and Hall, 2005; Mattila and Lappalainen, 2008; Ridley, 2011; Small et al., 2002; Vicente-Manzanares et al., 2009). Within the cell body,

actin filaments are arranged as anti-parallel ‘stress’ fibers, which create the contractile forces required during cell movement (Katoh et al., 2001).

In addition to actin polymerisation being a vital aspect of cell migration, cell-substratum adhesions also play an important role. Integrin-mediated signalling helps regulate cell attachment to the ECM during migration (Huttenlocher and Horwitz, 2011). Integrins are transmembrane glycoprotein receptors, which are composed of an α and β subunit. They couple the cytoskeleton, via adapter proteins, to components of the ECM including collagens, laminins and fibronectin (Hood and Cheresh, 2002). Integrins cluster in a small area, developing into a focal complex, which mature into more stable focal adhesions. These adhesions remain in contact with the ECM while the cell moves, until they reach the rear of the cell, where they disassemble. Disassembly is mediated either through enzymatic action (calpain, for example) or a reduction in integrin affinity for ECM components. This allows the trailing edge to retract and for the cell to move forward (Friedl and Wolf, 2003; Hood and Cheresh, 2002; Huttenlocher and Horwitz, 2011; Lauffenburger and Horwitz, 1996). Integrins are ubiquitously expressed, but overexpression and aberrant behaviour is observed in some cancer types, promoting metastasis, including within PDAC (Hosotani et al., 2002). Taken together, it is clear that cell motility is a highly complex process, which is highly regulated. Orchestrating the process is a wide range of receptor, bundling, capping, adhesion and motor proteins (Ananthakrishnan and Ehrlicher, 2007); however, the formation of these cellular protrusions is a key process in cell migration, which is true of both normal and cancerous cells.

1.3.2 Metastasis

Although there are many cellular processes that require cell migration, this study has focused on pancreatic cancer cell migration. In cancer, deregulated cell migration results in metastasis of tumour cells to distant sites around the body. In these instances, cells gain an invasive capacity, changing from a benign to a malignant state. In order for cells to metastasise, there are a number of key steps: detachment from the primary tumour, local invasion, intravasation into the blood or lymphatic system, dissemination and extravasation and subsequent outgrowth at distal, secondary sites (**figure 1.5**) (Valastyan and Weinberg, 2011; Yilmaz and Christofori, 2009). As stated, they must first break away from the primary tumour, often undergoing EMT. A loss of E-cadherin is characteristic of this process, leading to the loss of intercellular junctions and is initiated through the repression of transcriptional regulators such as Snail and Twist (Brabek et al., 2010; Liu et al., 2008b), which are known to be upregulated in PDAC (Dangi-Garimella et al., 2012). This then enables cells to adopt a more motile phenotype (Yilmaz and Christofori, 2009). In order to invade locally, cancer cells must breach the basement membrane, a specialised ECM known to organise epithelial tissues and maintain tissue architecture (Yurchenco, 2011). Cancer cells are thought to use similar molecular programmes that are used during development to produce gaps in the basement membrane in order to spread, such as proteolytic degradation (Hotary et al., 2006; Ihara et al., 2011). MMPs are the most prominent proteinases that allow cancer cells to metastasise and while basement membrane and ECM degradation is their primary function, they also regulate pathways associated with cell growth and

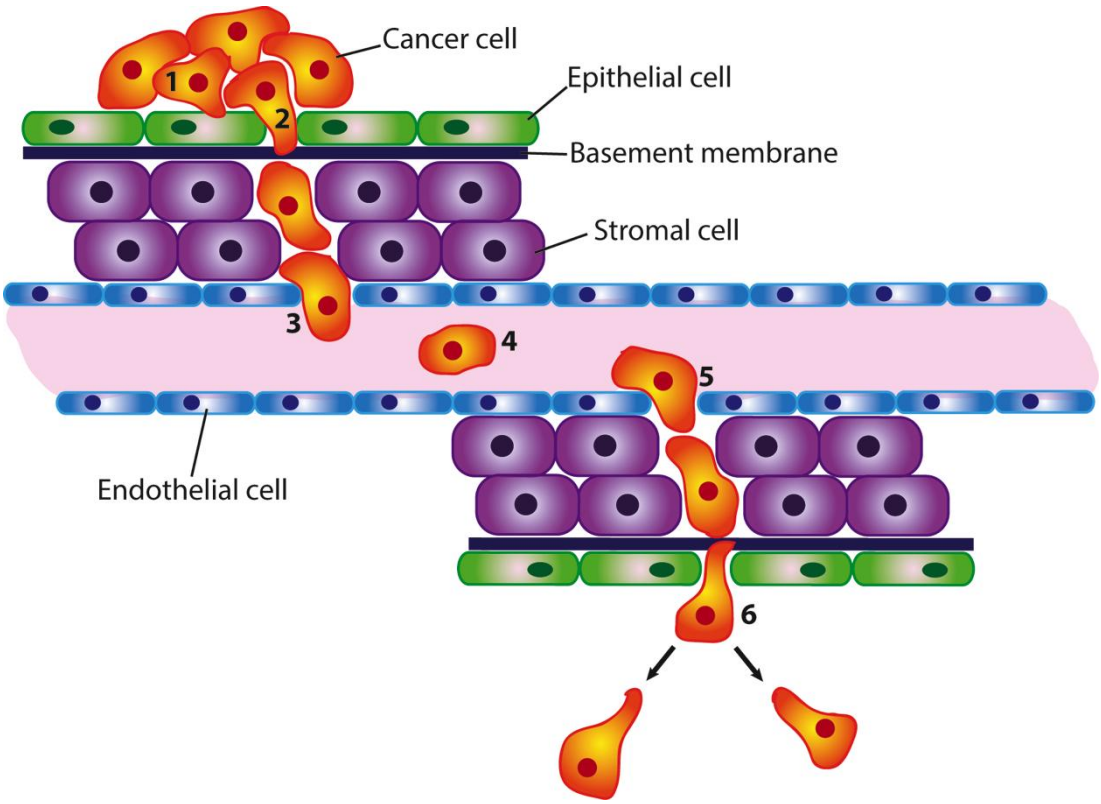


Figure 1.5: Metastatic process. Metastasis requires several steps including (1) reduced adhesion to neighbouring cells and detachment from the primary tumour. (2) Local invasion and invasion through the basement membrane into the stroma. (3) Intravasation into the vasculature. (4) Cancer cells travel around the body, often protected from immune cells by platelets. (5) Cancer cells get trapped and bind to receptors on endothelial cells and extravasate. (6) Cancer cells then invade, proliferate and subsequently form secondary tumours at distal sites around the body.
Figure adapted from: (Schroeder et al., 2012)

angiogenesis (Kessenbrock et al., 2010). Overexpression of several MMPs, including MMP2 and MMP9 is well documented in pancreatic cancer (Haq et al., 2000; Jimenez et al., 2000; Zervos et al., 1999; Zhang et al., 2011b).

Once the basement membrane has been breached, cancer cells are then able to invade locally and disseminate from the primary tumour and enter the stroma (Valastyan and Weinberg, 2011). Here they interact with stromal cells, which can contribute to tumour progression (see **section 1.1.6**). Cancer cells can migrate by a variety of different mechanisms; either by amoeboid or mesenchymal type movement, or collectively in clusters or strands. Attempted inhibition of one mode of migration can result in cancer cells converting to another form (Brabek et al., 2010; Friedl and Wolf, 2003). Cells moving via mesenchymal type migration have a characteristic elongated, fibroblast-like appearance. Molecules such as MMPs are recruited to aid in developing a pathway through the dense ECM meshwork (Brabek et al., 2010). In amoeboid migration, cells remain in a more rounded shape and movement is generated through repeated cycles of expansion and contraction of the cell body. Cells are extremely deformable and are able to squeeze through vey narrow regions within the matrix. This type of cell migration requires the activation of the Rho/ROCK pathway and subsequent phosphorylation of myosin light chain-2 (MLC-2) (Brabek et al., 2010; Sahai and Marshall, 2003; Webb and Horwitz, 2003). Collective cell migration can take place in the form of sheets/strands of cancer cells, which stay in contact with the primary tumour but invade into the local area, or in the form of clusters/groups of cells that detach from the primary tumour and often enter

the blood or lymphatic system in order to metastasis (Friedl and Wolf, 2003; Ilina and Friedl, 2009).

Entry of cancer cells into the stroma provides opportunities to access the circulation, which is achieved through a process called intravasation (Valastyan and Weinberg, 2011). Compared to other tumours, PDACs are poorly vascularised, making a very hostile environment that the cancer cells must adapt to; this is thought to contribute to the intense chemoresistance observed in PDAC (Bartholin, 2012). Lymphatic metastasis is an early even in PDAC progression and is an independent poor prognosis factor (Xiao et al., 2014). Indeed, the angioregulatory growth factor, angiopoietin-2 (Ang-2), was found expressed in transformed PDAC cell samples. This was found to promote lymphatic metastasis through binding to the corresponding receptor on blood and lymphatic epithelium (Schulz et al., 2011). Accumulating evidence supports the theory that the accumulating mutations in PDAC and microenvironment lead to the formation of metastasis-initiating cells (MIC), which initiate lymphatic metastasis through chemokine guidance mechanisms (Xiao et al., 2014). The ability of cancer cells to evade anoikis is essential for survival in the circulatory systems. Several mechanisms have been proposed to promote anchorage independent growth in PDAC. This includes upregulation of Wnt/β-catenin signalling (Arensman et al., 2014), expression of protein kinase D-1 (PKD-1) (Ochi et al., 2011), overexpression of the architectural transcription factor, high-mobility group A1 (HMGA1)(Liau et al., 2007) and expression of dual endothelin1/VEGF signal peptide receptor, DEspR (Herrera et al., 2014). Furthermore, it is thought that PAK4

(see **section 1.4**) could promote anchorage independent growth in PDAC (Kimmelman et al., 2008). Cancer cells must then arrest within the vessels and extravasate in order to form distal metastases (Gupta and Massague, 2006). In PDAC expression of neuropilin-2 (NRP-2) has been shown to mediate vascular adhesion and extravasation through interaction with α5 integrin on endothelial cells (Cao et al., 2013). The metastasis-associated gene S100P has also been shown to facilitate both intravasation and extravasation in PDAC *in vitro* and *in vivo* using a zebrafish embryo model (Barry et al., 2013), which is mediated through cytoskeletal changes and regulation of cathepsin D (Whiteman et al., 2007). Once cells extravasate, if the secondary sites microenvironment is conducive to supporting cancer cell growth, micrometastases will be generated; these can then proliferate and form macroscopic lesions (Saxena and Christofori, 2013). The most common secondary sites for metastasis in PDAC are the liver and peritoneal cavity; less common sites include the lung, bone and brain (Borad et al., 2009). These findings are recapitulated in genetically engineered mouse models of PDAC using Pdx1-Cre/Lox-Stop-Lox (LSL)-Kras or p48Cre/LSL-Kras mice. In these models the LSL regulates active K-ras and additional deletions in various tumour suppressor genes are often incorporated (Grippo and Tuveson, 2010; Hingorani et al., 2003; Saxena and Christofori, 2013). The ability of cancer cells to invade and metastasise is an area of extensive research and studies of factors, proteins and signalling networks contributing to this process are continuing to expand knowledge. One such group of proteins shown to contribute heavily to cell migration and metastasis are the family of p21-activated kinases (PAKs).

1.4 PAKs

1.4.1 PAK overview

The p21-activated kinases (PAKs) are serine/threonine kinases, of which there are six isoforms and are known to be downstream effectors of the Rho GTPases, Rac and Cdc42. They can be sub-divided into two groups, based on structural similarities, sequence homology and regulation: group I PAKs (PAK1-3) and group II PAKs (PAK4-6) (Arias-Romero and Chernoff, 2008; Bokoch, 2003; Jaffer and Chernoff, 2002). Nearly all eukaryotes, with the exception of plants, encode one or more of the PAK genes and various experimental systems have been utilised in order to determine the multitude of PAK functions (Hofmann et al., 2004). It is well documented that PAKs act as effectors for a wide range of signalling pathways. Activated PAKs are able to relocalise to the nucleus, where they manipulate gene transcription (Li et al., 2012; Tao et al., 2011) or to the leading edge of cells to influence cytoskeletal organisation and contribute to cellular motility (Arias-Romero and Chernoff, 2008; Bagrodia and Cerione, 1999; Sells and Chernoff, 1997). In addition, PAKs are known to play a role in hormone signalling (Eswaran et al., 2008; Lee et al., 2002; Rayala et al., 2006; Schrantz et al., 2004), apoptosis/cell survival (Cotteret et al., 2003; Ong et al., 2011; Schurmann et al., 2000; Ye et al., 2011) and changes in cell morphology (Cau et al., 2001; Manser et al., 1997; Zeng et al., 2000).

Of the group II PAKs, PAK4 was the first to be identified and is the most widely studied. It was originally identified as a cytoskeletal regulatory protein, controlling filopodia formation downstream of active Cdc42 (Abo et al., 1998).

However, more recent research has also highlighted its requirement in embryonic development, with PAK4 knockout in mice resulting in embryonic lethality (embryonic day 11.5) (Qu et al., 2003). Moreover, the number of substrates and binding partners for PAK4 is rapidly and continually expanding, it has been found to be fundamental in a plethora of cellular processes, highlighting PAK4 as a central signalling molecule (Dart and Wells, 2013). Indeed, because PAK4 plays a key role in cell signalling, it is not surprising that deregulation of PAK4 activity contributes to a number of disease states, including oncogenic transformation and cancer progression (Kumar et al., 2006; Kumar and Vadlamudi, 2002; Wells and Jones, 2010). Although an overview of all PAK members will be provided, PAK4 will remain the main focus.

1.4.2 PAK4 expression and localisation

It is known that PAK4 is required for embryonic development, being important for foetal heart and nervous system development (Qu et al., 2003; Tian et al., 2009; Tian et al., 2011b). PAK4 has a much lower level of protein expression in adult tissues (Minden, 2012); it is still found ubiquitously expressed in a wide range of tissue, but detected at particularly high levels in the prostate, testis and colon (Abo et al., 1998; Callow et al., 2002). Inactive PAK4 is predominantly found in the perinuclear region, however, coexpression with active Cdc42 leads to PAK4 localisation at the Golgi (Abo et al., 1998; Baldassa et al., 2010). Relocalisation of PAK4 to the cell periphery is observed in breast cancer cells and in response to HGF signalling in epithelial cells (Wells et al., 2002; Zhang et al., 2002). In

addition, PAK4 has been observed in cell substratum adhesion complexes in migrating macrophages and prostate cancer cell lines (Gringel et al., 2006; Wells et al., 2010); with recently identified nuclear import/export signals also contributing to the subcellular localisation of PAK4 (Li et al., 2012).

1.4.3 Structure of PAKs

PAKs show significant sequence homology to the Ste20 protein kinase, which is involved in the pheromone/mating factor pathway in the budding yeast, *Saccharomyces cerevisiae*. PAK related kinases have also been described in both *Drosopila* and *Caenorhabditis elegans* (Bagrodia and Cerione, 1999; Sells and Chernoff, 1997). Outside of the kinase domain, the sequences of PAKs and Ste20 protein kinase diverge except for a 60 amino acid sequence at the N-terminal, known as the p21-GTPase binding domain (GBD) (Sells and Chernoff, 1997). All PAKs contain this N-terminal GBD and a highly conserved C-terminal catalytic serine/threonine kinase domain (**figure 1.6**). Within the GBD of group I PAKs, there is also a Cdc42/Rac interactive binding region (CRIB), which overlaps with an autoinhibitory domain (AID) (Arias-Romero and Chernoff, 2008; Eswaran et al., 2007; Whale et al., 2011). With the exception of PAK5 (also known as PAK7) (Ching et al., 2003), the group II PAKs were not thought to contain an AID. However, more recently a putative AID has been identified in PAK4 (Baskaran et al., 2012), in a region that is conserved across all group II PAKs (Dart and Wells, 2013). In addition, all PAK proteins contain a variable number of core proline motifs (PxxP), which are putative binding sites for SH3 domain containing proteins, although no specific binding partners have

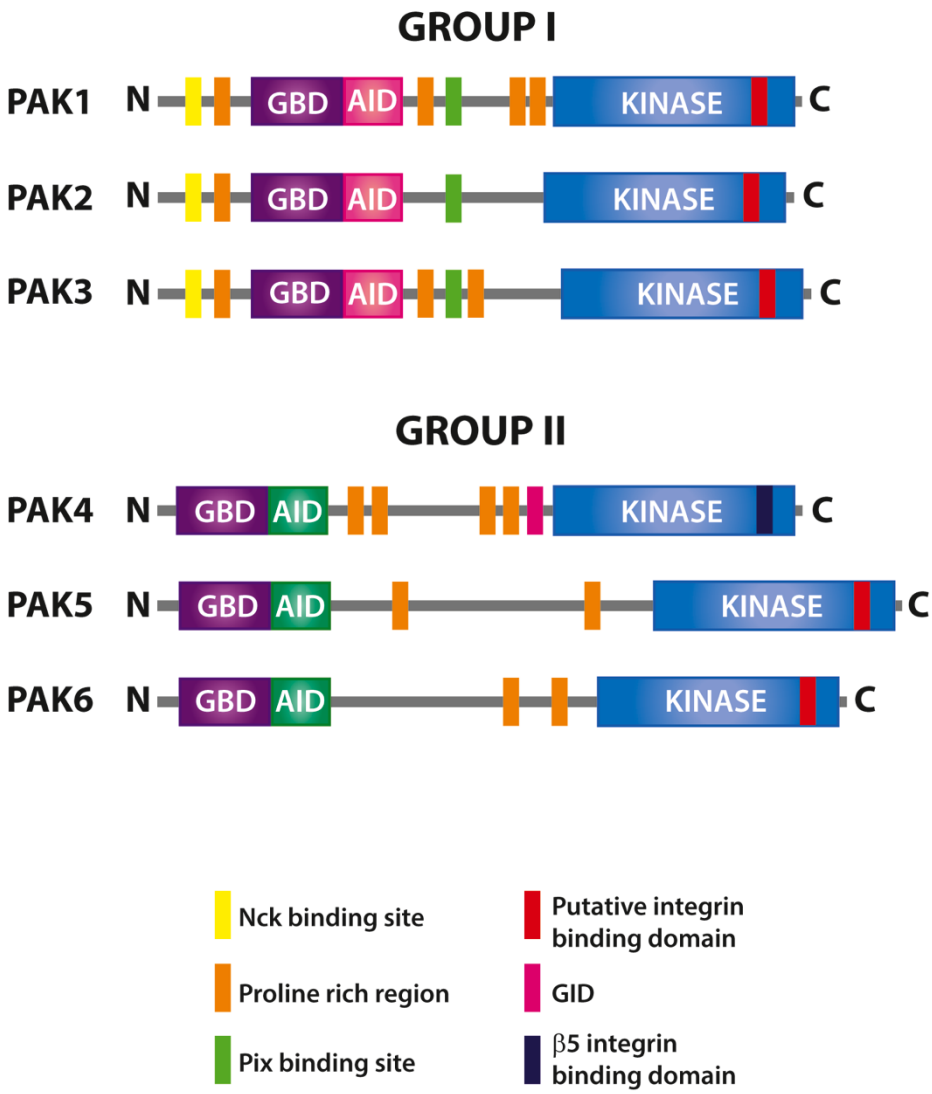


Figure 1.6: PAK structure. There are 6 PAK family kinases, which are divided into two group based on structural similarities and sequence homology. However, all PAKs have an N-terminal p21-GTPase binding domain (GBD) and a C-terminal kinase domain. Group I PAKs (PAK1-3) contain an autoinhibitory domain (AID), whereas group II PAKs contain a putative AID sequence. All PAKs contain a variable number of core PxxP motifs (proline rich regions), which are putative binding sites for SH3 domain containing proteins. Group I PAKs also contain a Nck1/2 binding site at the N-termini and a PIX binding site in the central region (indicated in yellow and green, respectively). Specific to PAK4 are a β5 integrin binding site (although this region is highly homologus among the other PAK family members) and a GEF-H1/Gab1 interacting domain (GID), which lies adjacent to the kinase domain.
Figure adapted from (Dart and Wells, 2013; King et al., 2014).

yet been identified. The sequence N-terminal to the GBD is smaller in group II PAKs, which in group I PAKs binds to the SH3 domains of Nck1/2 and Grb2. Another structural feature unique to group I PAKs is a non-classical SH3 domain, which is a specific binding site for the guanine nucleotide exchange factor, PAK-interacting exchange factor (PIX) (Arias-Romero and Chernoff, 2008; Bokoch, 2003; Chong et al., 2001; Howe, 2001; Whale et al., 2011; Zhao et al., 2000).

Specific to PAK4, upstream of the kinase domain, there is a GEF-H1/Gab1-interacting domain (GID), which binds to PDZ-RhoGEF (Barac et al., 2004) in addition to GEF-H1 (Callow et al., 2005) and Gab1 (Paliouras et al., 2009). PAK4 has also been shown to interact with the integrin αvβ5 via a binding site located within the kinase domain (Li et al., 2010c; Zhang et al., 2002), although it is hypothesised that all PAKs could potentially bind integrins. Although there are conserved structural motifs within the PAK family of proteins, all isoforms are structurally distinct and there is considerable variation. It is thought that this could provide substrate specificity, distinct functional roles and allow recognition of specific target sequences on binding partners (Arias-Romero and Chernoff, 2008; Rennefahrt et al., 2007; Wells and Jones, 2010). However, despite these structural differences PAK4 shares a number of substrates with PAK1 (Dart and Wells, 2013).

1.4.4 Regulation of PAK activity

Inactive group I PAKs form unphosphorylated homodimers, whereby the AID binds to the kinase domain and prevents its activity (Buchwald et al., 2001;

Eswaran et al., 2007; Parrini et al., 2002). Group I PAKs bind to both Rac and Cdc42 (King et al., 2014), this association between active Rac/Cdc42 with the GBD of group I PAKs disrupts the dimerisation; conformational changes destablilise the AID structure, leading to dissociation from the kinase domain and allowing autophosphorylation to occur (Arias-Romero and Chernoff, 2008; Chong et al., 2001). Autophosphorylation happens at several serine residues and at a single threonine residue (corresponding to Thr423 in PAK1), located within the activation loop of the kinase domain, which allows full kinase activity (Chong et al., 2001; Gatti et al., 1999; Wang et al., 2011).

In contrast to group I PAKs, binding of active Rho GTPases had no significant impact on PAK4 kinase activity (Abo et al., 1998). With the exception of PAK5 (Ching et al., 2003), it was originally thought that group II PAKs had no AID present and there has been much speculation over the regulation of PAK4 kinase activity. Recently, however, two possible mechanisms of PAK4 activity regulation have been put forward. The first identifies a potential AID in the N-terminal, composed of amino acids 20-68. This it is thought could hold PAK4 in an inactive conformation until binding of active Cdc42 leads to structural changes allowing PAK4 activation (Baskaran et al., 2012). The second study revolves around a newly identified pseudosubstrate, which contains a critical proline residue (R^{49}PKPLV). They concluded that this sequence was necessary and sufficient to inhibit PAK4 kinase activity, but also that there were potentially other regions that may facilitate complete inhibition. They noted binding of a SH3 domain-containing proteins such as Src was able to relieve autoinhibition (**figure 1.7**).

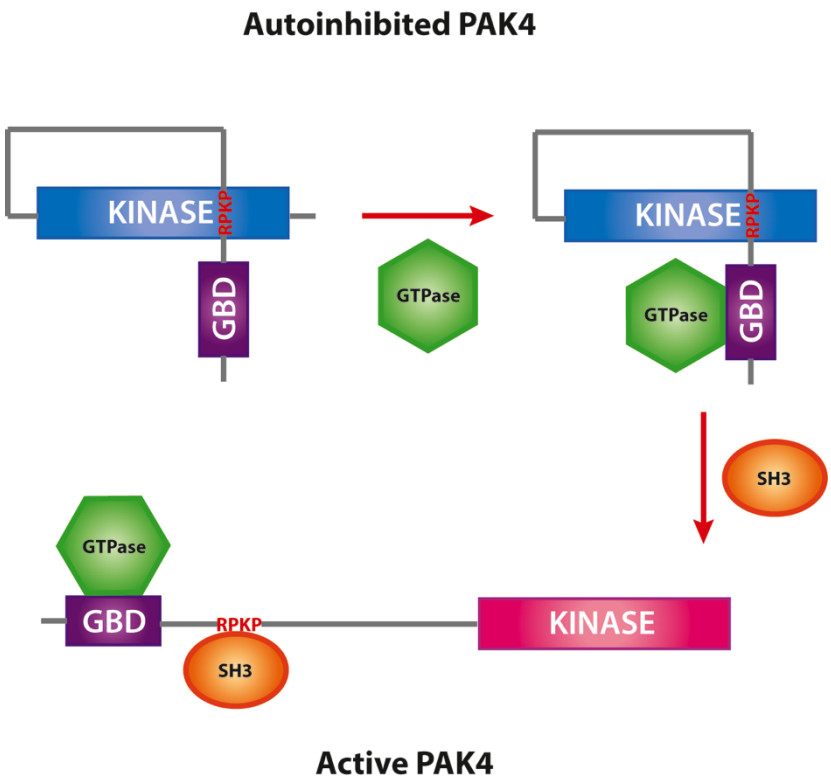


Figure 1.7: Mechanism of PAK4 autoinhibition. PAK4 has been identified as having an autoinhibitory pseudosubstrate (RPKP). It has been reported that GTPases binding to the GBD does not activate PAK4, but is important for subcellular localisation. However, a secondary signal from binding of a SH3 domain-containing protein causes a conformational change, releasing pseudosubstrate autoinhibition and allows kinase activity. This interaction may be through direct binding to the pseudosubstrate sequence or via another proline region within the N-terminus.
Figure adapted from: (Ha et al., 2012).

Additionally, they reported that autoinhibition was relieved upon mutation of this region (Ha et al., 2012), which compliments previous studies demonstrating that there is increased kinase activity in N-terminally truncated PAK4 (Wells et al., 2002). Further to the two studies, the entire AID sequence of PAK was elucidated through nuclear magnetic resonance (NMR) studies. It was found to comprise 20 residues with an N-terminal α-helix and the previously identified pseudosubstrate motif, which occupied the kinase cleft to achieve full autoinhibition (Wang et al., 2013). These studies do all conclude that PAK4 is likely to be constitutively phosphorylated at Ser474, a site previously used as a marker of PAK4 kinase activity; rather than being regulated by phosphorylation of residues within the activation loop, PAK4 activity is modulated via conformational changes mediated by N-terminal regions. They further postulate that this newly identified AID could be conserved across all group II PAKs, providing a common mechanism of autoregulation (Baskaran et al., 2012; Ha et al., 2012; Wang et al., 2013)

1.4.5 PAK4 and cell migration

PAK4 was originally identified as a cytoskeletal regulatory kinase, relocalising to the Golgi downstream of activated Cdc42 and inducing actin polymerisation and formation of filopodia in both endothelial cells and fibroblasts (Abo et al., 1998; Qu et al., 2001). Subsequent to this, PAK4 has been the focus of intense study and multiple cell migrational functions have been identified. It has been shown that overexpression of PAK4 leads to dissolution of stress fibers, a loss of focal adhesions and cell rounding (Barac et al., 2004; Qu et al., 2001; Wells et al., 2002), which is thought to be, at

least in part, due to the interaction of PAK4 with GEF-H1. Interaction between the two proteins, leads to phosphorylation GEF-H1 and inhibition of its stress fiber formation functions. In addition, this study provided a mechanistic insight into the role of PAK4/GEF-H1 interaction and crosstalk between microtubules and the actin cytoskeleton (Callow et al., 2005).

Cofilin is a regulator of actin filament disassembly; LIM-kinase (LIMK) has been shown to target cofilin, and through phosphorylation reverse cofilin-induced actin depolymerisation (Yang et al., 1998). PAK4 has been shown to interact and phosphorylate LIMK, enhancing its ability to phosphorylate cofilin to further inhibit actin disassembly (Ahmed et al., 2008; Dan et al., 2001). This is thought to be due to inhibition of Sling-Shot phosphatase-1L (SSH-1L) by PAK4, relieving the SSH-1L-induced down-regulation of LIMK (Soosairajah et al., 2005). Upregulation of LIMK activity by PAK4 is further enhanced by protein kinase D (PKD) mediated phosphorylation of PAK4 at Ser474 (Doppler et al., 2014; Spratley et al., 2011). In addition to the LIMK-mediated pro-migratory function of PAK4, it has been shown to regulate cell adhesion dynamics, which are a key component of cell migration. Both PAK4 knockout mice, and PAK4 depleted cells have an increase in the number of focal adhesions (Qu et al., 2003; Wells et al., 2010), which is thought to be linked to the reduction in PAK4-mediated phosphorylation of paxillin at Ser272 (a marker of adhesion dynamics); this phosphorylation is thought could promote adhesion disassembly (Wells et al., 2010; Zhang et al., 2008). It is known that PAK4 interacts with the cytoplasmic tail of integrin αvβ5 (Zhang et al., 2002), phosphorylating the β5 subunit at two sites (Ser759 and

Ser762), which are located within a membrane-proximal integrin beta5-SERS-motif (Li et al., 2010c). These phosphorylation events serve to accelerate integrin αvβ5 turnover within adhesions through inhibition of integrin αvβ5 clustering, connections to F-actin and preventing adhesion maturation (Li et al., 2010b), which is dependent on PAK4 kinase activity and promotes cell motility. PAK4 has also been shown to interact with the scaffold protein, Gab1, leading to relocalisation to the cell periphery (specifically within lamellipodia), an interaction that occurs downstream of c-Met, in response to HGF (Paliouras et al., 2009).

1.4.6 PAK4 and cancer

Overexpression and/or mutational activation of different PAK isoforms has been observed in many different tumour types, data suggest that aberrant PAK activity drives many cellular processes, which are central features of cancer (King et al., 2014; Radu et al., 2014). In this study, the mechanisms of PAK4-driven oncogenesis and its role in cancer progression are discussed. However, alterations of both group I and group II PAKs is summarised in **table 1.5**.

Cancer	PAK	Alteration	Key References
Bladder	PAK1	Genomic amplification	(Ito et al., 2007)
Breast	PAK1	Genomic amplification Increased phosphorylation Protein overexpression	(Balasenthil et al., 2004; Bostner et al., 2007; Stofega et al., 2004; Vadlamudi et al., 2000)
	PAK2	Increased phosphorylation Protein overexpression	(Li et al., 2011b; Stofega et al., 2004; Vadlamudi et al., 2000)
	PAK4	Genomic amplification Protein overexpression	(Callow et al., 2002; Liu et al., 2010b; Liu et al., 2008a; Minden, 2012; Rafn et al., 2012; Yu et al., 2009)
	PAK6	Protein overexpression	(Rafn et al., 2012)
Colorectal	PAK1	Protein overexpression	(Carter et al., 2004)
	PAK4	Genomic amplification	(Tabusa et al., 2013)
	PAK5	Protein overexpression	(Gong et al., 2009; Wang et al., 2010)
Endometrial	PAK1	Protein overexpression	(Lu et al., 2013a)
Gallbladder	PAK4	Protein overexpression	(Kim et al., 2008)
Gastric	PAK1	Protein overexpression	(Wu et al., 2014)
	PAK4	Protein overexpression	(Ahn et al., 2011; Zhang et al., 2012)
	PAK5	Protein overexpression	(Gu et al., 2013)

Glioblastoma	PAK1	Increased phosphorylation	(Aoki et al., 2007)
Glioma	PAK4	Protein overexpression	(Kesanakurti et al., 2012)
	PAK5	Protein overexpression	(Han et al., 2013)
Hepatocellular carcinoma	PAK1	Genomic amplification Protein overexpression	(Ching et al., 2007)
	PAK2	Increased phosphorylation	(Sato et al., 2013)
	PAK5	Gene amplification	(Fang et al., 2014)
	PAK6	Protein overexpression	(Chen et al., 2014)
Kidney	PAK1	Increased phosphorylation	(O'Sullivan et al., 2007)
Lung	PAK1	Protein overexpression	(Ong et al., 2011)
	PAK4	Protein overexpression	(Callow et al., 2002)
	PAK5	Gain of function gene mutation	(Fawdar et al., 2013)
Melanoma	PAK1	Genomic amplification Protein overexpression	(Ong et al., 2013)
Neuroendocrine	PAK3	Protein overexpression	(Liu et al., 2010a)
Ovarian	PAK1	Genomic amplification Increased phosphorylation Protein overexpression	(Brown et al., 2008; Davidson et al., 2008; Schraml et al., 2003; Siu et al., 2010b)

	PAK2	Increased phosphorylation	(Siu et al., 2010b)
	PAK4	Genomic amplification Protein overexpression	(Davis et al., 2013; Siu et al., 2010a)
	PAK5	Protein overexpression	(Li et al., 2013)
Pancreatic	PAK1	Protein overexpression Reduced protein expression	(Zhou et al., 2014) (Han et al., 2014)
	PAK4	Genomic amplification	(Chen et al., 2008; Kimmelman et al., 2008; Mahlamaki et al., 2004)
Prostate	PAK1	Protein overexpression	(Goc et al., 2013)
	PAK2	Protein overexpression	(Jiang et al., 2014)
	PAK4	Protein overexpression	(Ahmed et al., 2008; Park et al., 2013; Wells et al., 2010; Whale et al., 2013)
	PAK6	Protein overexpression	(Fram et al., 2014; Kaur et al., 2008; Zhang et al., 2010)
Squamous cell carcinoma	PAK1	Protein overexpression	(Chow et al., 2012)
	PAK4	Genomic amplification	(Begum et al., 2009; Zanivan et al., 2013)
T-cell lymphoma	PAK1	Genomic amplification	(Mao et al., 2003)

Table 1.5: PAKs in cancer. Table demonstrating the range of cancers different PAK isoforms are involved in and the type of alterations involved. Table adapted from King *et al*, 2014 including some newly published data. Of note are the different studies of PAK1 involvement in pancreatic cancer, demonstrating the complexities involved in this disease and the requirement for further investigation.

With regards to PAK4, it is the most widely studied group II PAK, especially in respect to cancer. The PAK4 gene maps to a region on chromosome 19 (19q13.2), which is commonly amplified in cancer (Begum et al., 2009; Chen et al., 2008; Minden, 2012; Parsons et al., 2005; Yu et al., 2009). PAK4 has been shown to be overexpressed or genetically amplified and/or mutated in a number of cancer cell lines and tumour samples including breast (Callow et al., 2002; Liu et al., 2010b; Minden, 2012; Wong et al., 2013), ovarian (Davis et al., 2013; Siu et al., 2010a), colon (Parsons et al., 2005; Tabusa et al., 2013), prostate (Ahmed et al., 2008; Park et al., 2013; Wells et al., 2010; Whale et al., 2013), gastric (Ahn et al., 2011), lung (Callow et al., 2002), glioma (Kesanakurti et al., 2012), gallbladder (Kim et al., 2008), squamous cell carcinoma (Begum et al., 2009; Zanivan et al., 2013) and pancreatic (Chen et al., 2008; Kimmelman et al., 2008; Mahlamaki et al., 2004).

It is known that PAK4 plays a key role in cellular proliferation in the developing embryo (Qu et al., 2003; Tian et al., 2009; Tian et al., 2011b), but also controlling key aspects of normal cellular proliferation. PAK4 regulates both the G_1 (Nekrasova and Minden, 2011) and G_2/M phase (Bompard et al., 2010) of the cell cycle and is important in spindle positioning during mitosis (Bompard et al., 2013). However, the impact of PAK4 on proliferation has also been reported in cancer, which allows cells to rapidly expand in number. PAK4 was found to regulate the expression of both cyclin D1 and CDC25A in ovarian cancer cells, with shRNA-mediated depletion of PAK4 resulting in a significant reduction in proliferation (Siu et al., 2010a). It is also thought that PAK4 could balance the expression of CDK6 and p16 (a tumour suppressor

that inhibits CDK6) to favour an increased proliferative rate in choriocarcinoma cells (Zhang et al., 2011a). Further evidence shows that increased PAK4 expression in gastric cancer correlated with cell cycle progression via PAK4 phosphorylation of Smad2/3 and subsequent inhibition of TGF-β1 signalling (Wang et al., 2014). Conversely, cell cycle arrest is observed in PAK4 knockdown laryngeal carcinoma cells through activation of the ATM/Chk1/2/p53 pathway (Sun et al., 2013). Additionally, PAK4 knockdown in colon carcinoma cell lines resulted in significantly reduced proliferation; this was, however, found not to involve either the MAPK or PI3K/Akt signalling pathways and was irrespective of PAK4 protein levels (Tabusa et al., 2013). These data provide strong evidence to support a role for PAK4-driven cell cycle progression in tumourogenesis.

Evasion of apoptosis is another hallmark of cancer and PAK4 has been shown to promote cell survival through both kinase-dependent and kinase-independent mechanisms (King et al., 2014). Cells overexpressing either wild type or constitutively active PAK4 were able to avoid apoptosis after exposure to pro-apoptotic stimuli through prevention of caspase activation (Gnesutta et al., 2001). PAK4 protection from apoptosis requires both kinase-independent antagonism of caspase 8 activation and prevents recruitment to death domain receptors (Gnesutta and Minden, 2003) and PAK4 kinase-dependent phosphorylation of the proapoptotic protein, Bad (Gnesutta and Minden, 2003). Cells lacking PAK4 are more susceptible to apoptosis, with cells requiring PAK4 to activate TNF-α mediated pro-survival pathways (Li and Minden, 2005) Additionally, PAK4 has been found to associate with

keratinocyte growth factor (KGF) receptor, which is an important protective factor for epithelial cells. Expression of a dominant-negative PAK4 blocks KGF-mediated inhibition of caspase 3 activation (Lu et al., 2003), which further demonstrates a role for PAK4 in cell survival mechanisms. Furthermore, PAK4 gene amplification observed in ovarian cancer has recently been shown to be essential for cell viability (Davis et al., 2013).

PAK4 is also heavily implicated in cancer cell metastasis, a process that is reliant on reorganisation of the actin cytoskeleton. PAK4 is a known cytoskeletal regulatory kinase (see **section 1.4.5**). PAK4/LIMK interactions are known to contribute to cell migration (Dan et al., 2001) and this has been shown to be downstream of HGF in prostate cancer cells (Ahmed et al., 2008). Indeed, PAK4 depleted prostate cancer cells are less responsive to HGF, demonstrating a significant reduction in cell migration (Wells et al., 2010). This PAK4/LIMK signalling has also been shown to contribute to gastric cancer cell migration *in vitro*. LIMK phosphorylation by PAK4 is enhanced via interaction of DiGeorge critical region 6L with the PAK4 kinase domain (Li et al., 2010a). Additional pro-migratory signalling of PAK4 in gastric cancer metastasis is mediated through PAK4 dependent phosphorylation of superior cervical ganglia 10 (SCG10), a microtubule destabiliser, at Ser50. This serves to regulate microtubules and promote gastric cancer cell migration for *in vitro* and in a xenograft mouse model (Guo et al., 2014b). Further to the role of PAK4 in normal cell adhesion dynamics via integrins, PAK4 was shown to contribute to focal adhesion turnover and directional cell migration in prostate cancer through interaction with a β1

integrin/Trop-2 complex (Trerotola et al., 2013). PAK4 enhances endometrial cell invasion and PAK4 knockdown in both endometrial and glioma cells produces a significantly reduced migratory phenotype (Kesanakurti et al., 2012; Kim et al., 2013). Overexpression studies of PAK4 in ovarian cancer demonstrate the ability of PAK4 to enhance ovarian cancer cell migration in a c-Src/MEK-1/MMP-2 kinase-dependent manner, with the level of c-Src and MMP-2 expression correlated with PAK4 knockdown (Siu et al., 2010a). In addition, in the MCF10 breast cancer progression cell series, the level of PAK4 expression is observed as correlating with tumourogenicity in the cell lines (So et al., 2012). PAK4 is known to trigger transformation of mammary epithelial cells (Liu et al., 2010b), with PAK4 depletion in MDA-MB-231 breast cancer cells leading to both reduced migration *in vitro* and decreased tumour formation in nude mice *in vivo* (Wong et al., 2013) and also contributing to oncogene ErbB2-induced breast cancer cell invasiveness (Rafn et al., 2012). There was a marked decrease in the invasive capacity of PAK4-depleted skin cancer cell lines (A431 and SCC9) in a collagen-based 3D organotypic model, which may be due to ablation of PAK4-mediated phosphorylation of RAF1 and Src, at Ser43 and Ser17 respectively (Zanivan et al., 2013). Overexpression of PAK4 has also been documented in hepatocellular carcinoma tissue; this correlates with the known pro-metastatic protein, CDK5 kinase regulatory subunit-associated protein 3 (CDK5RAP3), which also enhanced PAK4 kinase activity (Mak et al., 2011). Interestingly, PAK4 has also been shown to be overexpressed in pancreatic cancer (Chen et al., 2008; Mahlamaki et al., 2004). Constitutively active PAK4 increases pancreatic duct cell invasion and siRNA-mediated depletion

of PAK4 in the highly invasive PaTu8988T PDAC cell line resulting in both a reduction of anchorage-independent growth and a decreased migratory phenotype (Kimmelman et al., 2008). However, the mechanisms of PAK4-mediated invasion and pro-migratory signalling in PDAC have yet to be elucidated. With the wealth of data published, it strongly implicates the effects of aberrant PAK4 signalling and its key role in invasive oncogenesis.

1.5 The project

1.5.1 Hypothesis

PAK4 is overexpressed in pancreatic cancer cell lines and in primary tumour samples. In addition, KRAS gene mutations occur frequently in pancreatic cancer; it is thought that these mutations could lead to PAK4 activation. HGF and its receptor c-Met are also upregulated in pancreatic cancers, these in combination with oncogenic K-ras lead to continual signalling of downstream pathways, including the PI3K pathway. PAK4 has been shown to be activated downstream of PI3K in an HGF dependant manner, leading to increased cell migration. In addition LY294002, a phosphoinositide 3-kinase (PI3K) inhibitor, inhibits HGF-induced PAK4 kinase activation (Wells et al., 2002). PAK4 has also been shown to be amplified in PDAC cell lines and tumour samples and that oncogenic K-ras can activate PAK4 (Chen et al., 2008). It was therefore hypothesised that PAK4 could lie within the PI3K pathway in pancreatic cancer downstream of K-ras and/or HGF/c-Met to promote pancreatic cancer cell migration and invasion.

1.5.2 Aims of the project

The aim of this project was to investigate the importance of PAK4 signalling in pancreatic cancer cell invasion, and the pathways involved in its activation and downstream effector functions. To facilitate the studies of PAK4 biology, knockdown cell lines would be used to demonstrate a requirement for PAK4 in pancreatic cancer cell migration downstream of HGF. This would be done both in random 2D migration assays and using an already established organotypic model of pancreatic cancer in order to study 3D invasion. In

parallel, PAK4 interacting partners, relevant to the pathways involved in PDAC progression will be isolated using GST pulldown assays. These interactions will be further explored using pharmacological inhibition of PI3K in order to determine if the effects of PAK4 knockdown are phenocopied. Finally, potential downstream effectors of PAK4 signalling in PDAC will also be investigated in order to provide a additional insight as to the nature of PAK4 signalling in pancreatic cancer.

Chapter 2
Materials and Methods

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 General Materials

10X Dulbecco's modified Eagle's medium (DMEM) (**VWR International, UK**)
2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) (**Cayman Chemical, USA**)
2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris)-Base/Hydrochloric acid (HCL) (**Sigma-Aldrich, UK**)
3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (**Sigma-Aldrich, UK**)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (**GIBCO®, Invitrogen, UK**)
4', 6-diamidino-2-phenylindole (DAPI) (**Sigma-Aldrich, UK**)
Acrylamide (30%) (**Severn Biotech Ltd, UK**)
Agarose (**Invitrogen, UK**)
Ammonium persulfate (APS) (**Sigma-Aldrich, UK**)
Ampicillin (**Sigma-Aldrich, UK**)
Aprotinin (**Sigma-Aldrich, UK**)
BD Matrigel^{TM} Matrix (**BD Biosciences, UK**)
Beta (β)-mercaptoethanol (**Sigma-Aldrich, UK**)
Bovine pituitary extract (BPE) (**GIBCO®, Invitrogen, UK**)
Bovine serum albumin (BSA) (**VWR International, UK**)
Bromophenol blue (**Bio-Rad, UK**)
Calcium phosphate transfection kit (**Invitrogen, UK**)
cOmplete, EDTA free protease inhibitor cocktail tabets (**Roche, UK**)
Concentrated rat tail collagen, type I (**BD Biosciences, UK**)
Coomassie brilliant blue (**Thermo Scientific, USA**)
Deoxyribonucleotide triphosphate (dNTPs) (**New England Biolabs UK**)
Dimethyl sulfoxide (DMSO) (**Sigma-Aldrich, UK**)
Dithiothreitol (DTT) (**Sigma-Aldrich, UK**)
DMEM F:12 media (**Lonza, UK**)
DMEM+GlutaMAX^{TM} (**GIBCO®, Invitrogen, UK**)

DNA ladder (**New England Biolabs, UK**)

Dulbecco's phosphate-buffered saline (DPBS) (**Lonza, UK**)

Epidermal growth factor (EGF) (**GIBCO®, Invitrogen, UK**)

Ethanol (**BDH Laboratory Supplies, UK**)

Ethidium bromide (**Thermo Fisher Scientific, UK**)

Ethylenediaminetetraacetic acid (EDTA) (**Sigma-Aldrich, UK**)

Fibronectin (**Sigma-Aldrich, UK**)

Fish skin gelatin (**Sigma-Aldrich, UK**)

FluorSave™ Reagent (**Calbiochem, UK**)

Foetal bovine serum (FBS) (**GIBCO®, Invitrogen, UK**)

Formalin solution (neutral buffered) (**Sigma-Aldrich, UK**)

Fuji Medical X-ray film, Super RX (**Fuji Film, Japan**)

Gateway™ BP Clonase™ Enzyme Mix (**Invitrogen, UK**)

Gateway™ LR Clonase™ Enzyme Mix (**Invitrogen, UK**)

Gentamycin (**Sigma-Aldrich, UK**)

Glacial acetic acid (**Sigma-Aldrich, UK**)

Glutathione Sepharose™ 4B Fast Flow beads (**GE Healthcare, UK**)

Gluteraldehyde (**Sigma-Aldrich, UK**)

Glycerol (**Sigma-Aldrich, UK**)

Glycine (**Sigma-Aldrich, UK**)

HCl (**VWR International, UK**)

HGF (Recombinant Human) (**R&D systems, USA**)

HiPerfect (**Qiagen Ltd, UK**)

Illustra™ GFX™ PCR DNA & Gel Band Purification Kit (**GE Healthcare, UK**)

Isopropanol (**VWR International, UK**)

Kanamycin (**Invitrogen, UK**)

Keratinocyte serum free media (KSFM) (**GIBCO®, Invitrogen, UK**)

L-Arabinose (**Sigma-Aldrich, UK**)

Lemo21 (DE3) chemiclally competent *E.coli* cells (**New England Biolabs, UK**)

Leupeptin (**Sigma-Aldrich, UK**)

Luria-agar (L-agar) (**Sigma-Aldrich, UK**)

Luria-broth (L-broth) tablets (**Sigma-Aldrich, UK**)

MAX Efficiency® DH5α™ Competent *Escherichia coli* (*E. coli*) cells (**Invitrogen, UK**)

Methanol (**VWR International, UK**)

Milk powder (**Marvel, UK**)

NEB-10 beta chemically competent *E. coli* cells (**New England Biolabs, UK**)

Nuclease-free water (**Fisher Scientific, UK**)

Nylon membrane (**Millipore, UK**)

Octylphenoxypolyethoxyethanol/Nonidet™ P40 (NP-40) (**Sigma-Aldrich, UK**)

One Shot® BL21-A1 Competent *E. coli* cells (**Invitrogen, UK**)

One Shot® TOP10 Chemically competent *E. coli* cells (**Invitrogen, UK**)

OptiMEM (**GIBCO®, Invitrogen, UK**)

Paraformaldehyde (PFA) (**Sigma-Aldrich, UK**)

pDEST™15 (**Invitrogen, UK**)

pDEST™207 (**Invitrogen, UK**)

Penicillin/Streptomycin (**Sigma-Aldrich, UK**)

Phenylmethylsulfonylfluoride (PMSF) (**Sigma-Aldrich, UK**)

Phosphate buffered saline (PBS) tablets (**Oxoid Limited, UK**)

Phusion DNA polymerase (**Finnzymes, UK**)

Phusion HF Buffer (**New England Biolabs, UK**)

Pierce® ECL western blotting substrate (**Thermo Scientific, USA**)

Precision Plus Protein™ All Blue Standards (**Bio-rad, UK**)

Purelink™ HiPure Plasmid Filter Maxi-prep kit (**Invitrogen, UK**)

Purelink™ HiPure Plasmid Filter Mini-prep kit (**Invitrogen, UK**)

Puromycin (**Sigma-Aldrich, UK**)

QIAfilter™ Plasmid Midi-prep kit (**Qiagen, UK**)

QuikChange™ Site-directed mutagenesis kit (**Stratagene, USA**)

Rat tail collagen, Type I (**BD Biosciences, UK**)

Rat tail collagen, Type I (**Millipore, UK**)

Roswell Park Memorial Institute (RPMI)-1640 medium (**GIBCO®, Invitrogen, UK**)

Sodium borohydride (**Sigma-Aldrich, UK**)

Sodium chloride (NaCl) (**Sigma-Aldrich, UK**)

Sodium dodecyl sulphate (SDS) (**Sigma-Aldrich, UK**)

Sodium fluoride (NaF) (**Alfa Aesar, UK**)

Sodium hydroxide (NaOH) (**Sigma-Aldrich, UK**)

Sodium orthovanadate (Na_3VO_4) (**Sigma-Aldrich, UK**)

Sodium pyrophosphate (**BDH Chemicals, UK**)

Spectinomycin (**Sigma-Aldrich, UK**)

Sucrose (**Sigma-Aldrich, UK**)

Tetramethylethylenediamine (TEMED) (**Sigma-Aldrich, UK**)

Tri-sodium citrate dihydrate (**Sigma-Aldrich, UK**)

Tris acetate (**Sigma-Aldrich, UK**)

Tris-base (**Sigma-Aldrich, UK**)

Tris-EDTA (TE) Buffer (**Invitrogen, UK**)

TritonX-100 (**VWR International, UK**)

Trypsin/EDTA (**GIBCO®, Invitrogen, UK**)

Tween20 (**VWR International, UK**)

Whatman PROTRAN nitrocellulose membrane (**Perkin Elmer, USA**)

X-tremeGENE HP transfection reagent (**Roche, UK**)

Xylene (**VWR International, UK**)

2.1.2 Buffers

Blocking solution: 5% w/v milk powder or 5% BSA in TBS-Tween

Coomassie blue stain: 50% v/v methanol, 10% v/v acetic acid, 0.025% w/v coomassie blue

Coomassie destain solution: 12.5% v/v isopropanol, 10% v/v acetic acid

DNA loading buffer: 40% w/v sucrose, 0.25% w/v bromophenol blue

Freeze down buffer: (for GST proteins): 50% v/v glycerol, 20mM Tris-HCl pH7.6, 100mM NaCl plus protease inhibitor cocktail

Harsh stripping buffer: 62.5mM Tris pH6.8, 2% w/v SDS, 100mM β-mercaptoethanol

Laemmli buffer (2X): 100mM Tris-HCl pH6.8, 4% w/v SDS, 20% v/v glycerol, 0.2% w/v bromophenol blue, 1:50 β-mercaptoethanol

Laemmli buffer (6X): 375 mM Tris-HCl pH 6.8, 6% SDS, 48% glycerol, 9% β-mercaptoethanol, 0.03% bromophenol blue

Mild stripping buffer: 25mM Glycine pH2, 1% w/v SDS

NP-40 lysis buffer: 0.5% v/v NP-40, 30mM sodium pyrophosphate, 50mM Tris-HCl pH7.6, 150mM NaCl, 0.1mM EDTA plus protease inhibitor cocktail

Protease inhibitor cocktail: 50mM NaF, 1mM Na_3VO_4, 1mM PMSF, 10µg/ml leupeptin, 1µg/ml aprotinin and 1mM DTT

Pulldown lysis buffer: 20mM Tris-HCl pH7.6, 150mM NaCl, 2mM EDTA, 0.01% v/v TritonX-100, 10% v/v glycerol plus protease inhibitor cocktail

Pulldown wash buffer: 20mM Tris-HCl pH7.6, 300mM NaCl, 2mM EDTA, 1% v/v TritonX-100, 10% v/v glycerol plus protease inhibitor cocktail

SDS-PAGE running buffer (10X): 250mM Tris-base, 1.92M Glycine, 1% w/v SDS. Dilute to 1X with distilled water for use.

SDS-PAGE transfer buffer (10X): 250mM Tris-base, 1.92M Glycine. Dilute to 1X with distilled water and methanol (to a final concentration of 20% v/v)

TBS-Tween: 25mM Tris-HCl pH7.6, 50mM NaCl, 0.1% v/v Tween 20

2.1.3 Plasmids

Construct	Vector backbone	Source
Myc:H-ras V12	pDONR223 Addgene, USA (31201)	Generated by author
Myc:K-ras V12	pDONR223 Addgene, USA (31200)	Generated by author
Myc:N-ras V12	pDONR^{TM}207 Kind gift from Dr. Matthias Krause, King's College London.	Generated by author
GFP:PAK4 RNAiRescue	pEGFP-C1 (Clontech, UK) modified for use in Gateway^{TM}Technology by Kerry Shea, King's College London	Generated by author
GFP:K-ras	pEGFP-C1 (Clontech, UK) modified for use in Gateway^{TM}Technology by Kerry Shea, King's College London	Generated by author
GFP:p85α	pEGFP-C1 (Clontech, UK) modified for use in Gateway^{TM}Technology by Kerry Shea, King's College London	Generated by author
GFP:p85α SH3	pEGFP-C1 (Clontech, UK) modified for use in Gateway^{TM}Technology by Kerry Shea, King's College London	Generated by author
GFP:p85α ΔSH3	pEGFP-C1 (Clontech, UK) modified for use in Gateway^{TM}Technology by Kerry Shea, King's College London	Generated by author

Table 2.1: Mammalian Plasmids

Construct	Vector backbone	Source
GST	pGEX 2A1	Kind gift from Professor Anne Ridley, King's College London.
GST:PAK4	pDEST^{TM}15	Andrew Whale
GST:PAK4PxxP$_8$	pDEST^{TM}15	Andrew Whale
GST:PAK4ΔPxxP$_8$	pDEST^{TM}15	Andrew Whale
GST:PAK4 P105,108A	pDEST^{TM}15	Generated by author
GST:PAK4 P287,289A	pDEST^{TM}15	Generated by author

Table 2.2: Bacterial Plasmids

2.1.4 Primers

Gene	5’ – 3’ Sequence
PAK4 F	ATGTTTGGGAAGAGGAAGAAGCGG
PAK4 F (Gateway)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGTTTGGGAAGAGGAAGCGG
PAK4 R (Gateway)	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATCTGGTGCGGTTCTGGCGAT
H-ras F	ATGACGGAATATAAGCTGGTGGTG
H-ras R	TCAGGAGAGCACACACTTGCAGCT
H-ras F (Gateway)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGACGGAATATAAGCTGGTGGTG
H-ras R (Gateway)	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGGAGAGCACACACTTGCAGCT
K-ras4B F	ATGACTGAATATAAACTTGTGGTA
K-ras4B R	TTACATAATTACACACTTTGTCTT
K-ras4B R (Gateway)	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACATAATTACACACTTTGTCTT
N-ras F (Gateway)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGACTGAGTACAAACTGGTGGTG
N-ras R (Gateway)	GGGGACCACTTTGTACAAGAAAGCTGGGTCT
PAK4 RNAiRes F	GACTACCAGCACGAGAACGTGGTCGAGATGTACAACAG
PAK4 RNAiRes R	CTGTTGTACATCTCGACCACGTTCTCGTGCTGGTAGTC
P105,108A F	GACAGCGCGCCGCCGGCCGCCC
P105,108A R	GGGCGGCCGGCGGCGCGCTGTC
P287,289A F	CCTGGGCCCGCTGGCGCCCGCTCAC
P287,289A R	GTGAGCGGGCGCCAGCGGGCCCAGG

Table 2.3: Primers
N.B. Gateway denotes primers that contain the specific *att* sequences, which lies before the template specific sequence within the primer.

2.1.5 Antibodies

Antibody	Species	Company	Dilution for IF/IHC	Dilution for WB
AKT	Rabbit	Cell Signaling Technology	-	1:1000
β-catenin	Rabbit	Sigma-Aldrich	1:100	1:1000
c-Myc (9E10)	Mouse	Santa Cruz Biotechnology	-	1:500
Claudin1 (JAY.8)	Rabbit	Invitrogen	1:250	-
Cleaved Caspase3	Mouse	R&D Systems	1:100	-
Cytokeratin WSS	Rabbit	Dako	1:500	-
E-cadherin (HECD-1)	Mouse	Abcam	1:100	1:1000
ERK1/2	Rabbit	Cell Signaling Technology	-	1:1000
Gab1	Rabbit	Millipore	-	1:1000
GAPDH	Mouse	Millipore	-	1:10,000
GFP (7.1 and 13.1)	Mouse	Roche	-	1:1000
GST	Goat	GE Healthcare	-	1:5000
K-ras-2B (C19)	Mouse	Santa Cruz Biotechnology	-	1:500
Keratin18 (LDK18)	Mouse	A kind gift from Prof. E.B. Lane, University of Dundee	1:1000	-
Keratin8 (LE41)	Mouse	A kind gift from Prof. E.B. Lane, University of Dundee	1:100	-
Ki-67	Rabbit	Novocastra	1:200	-
Met (C-12)	Rabbit	Santa Cruz Biotechnology	-	1:500

N-cadherin (32)	Mouse	BD Transduction Laboratories	1:100	-
Occludin (OC-3F10)	Mouse	Invitrogen	1:250	-
p53	Mouse	Santa Cruz Biotechnology	-	1:100
PAK1	Rabbit	Cell Signaling Technology	-	1:1000
PAK2	Rabbit	Cell Signaling Technology	-	1:1000
PAK3	Rabbit	Cell Signaling Technology	-	1:1000
PAK4	Rabbit	In house	-	1:1000
PAK4/6	Rabbit	Cell Signaling Technology	-	1:1000
PAK5	Rabbit	In house	-	1:500
PAK6	Rabbit	Calbiochem	-	1:500
Paxillin (349)	Mouse	BD Transduction Laboratories	1:50	-
Phospho-AKT (Ser473)	Rabbit	Cell Signaling Technology	-	1:1000
Phospho-ERK1/2 (p44/42 MAPK, Thr202/Tyr204) (E10)	Mouse	Cell Signaling Technology	-	1:1000
Phospho-PAK4 (Ser474) PAK5 (Ser602) PAK6 (Ser560)	Rabbit	Cell Signaling Technology	-	1:1000
PI3 Kinase p85	Rabbit	Millipore	-	1:1000
Smooth muscle actin (1A4)	Mouse	Dako	1:300	1:100

Vinculin	Mouse	Sigma-Aldrich	1:100	-
ZO-1 (ZO1-1A12)	Mouse	Invitrogen	1:500	-

Table 2.4: Primary Antibodies

Antibody	Species	Company	Dilution for IF/IHC	Dilution for WB
HRP conjugated anti mouse	Goat	Dako	-	1:2000
HRP conjugated anti rabbit	Goat	Dako	-	1:2000
HRP conjugated anti goat	Rabbit	Dako	-	1:2000
AlexaFluor® 488 anti mouse	Goat	Invitrogen	1:200	-
AlexaFluor® 488 anti rabbit	Goat	Invitrogen	1:200	-
AlexaFluor® 568 anti mouse	Goat	Invitrogen	1:200	-
AlexaFluor® 568 anti rabbit	Goat	Invitrogen	1:200	-
AlexaFluor® Rhodamine Phalloidin		Invitrogen	1:500	-

Table 2.5: Secondary Antibodies

2.2 Methods – Molecular Biology

2.2.1 Generation of tagged constructs

The Gateway™ Technology system was used to generate tagged constructs. (N.B. This was only necessary for Ras constructs). Wild-type H-, K-, and N-ras constructs (a kind gift from Dr. Matthias Krause, King's College London) were used as templates in the production of Ras DNA flanked by *att*B sites; the addition of these sites is necessary in order to generate PCR products that were suitable for subsequent cloning into the Gateway™ vectors. Ras DNA flanked by the *att*B sequences were produced by PCR amplification using Phusion DNA polymerase. Each reaction was set up using 10µl reaction buffer, 500ng template DNA, 3% v/v DMSO, 200µM of deoxyribonucleotide triphosphate (dNTPs) and 500nM of each forward and reverse primer (see **table 2.3**). This was made up to a total reaction volume of 50µl, with nuclease-free water, in a sterile 0.2ml PCR tube. The PCR reaction was performed under the conditions described in **table 2.6**.

Number of cycles (& Process)	Temperature	Time
1 (initial denaturation)	95^{o}C	4 minutes
30	95^{o}C	30 seconds
	68^{o}C	30 seconds
1 (final extension)	68^{o}C	5 minutes

Table 2.6: PCR conditions for amplification of Ras constructs

2.2.2 Gel purification of DNA fragments

PCR products were resolved on a 1.3% w/v TAE agarose gel supplemented with 0.5µg/ml ethidium bromide to allow detection of DNA products. The DNA was then visualised under a low intensity UV light and could then be excised from the agarose gel using a scalpel. The DNA fragment was then purified using the Illustra^{TM} GFX^{TM} PCR DNA and Gel Band Purification kit according to the manufacturers protocol. In short, capture buffer was added to the sample to denature protein and/or dissolve agarose gel. The sample mixture was then applied to the Illustra^{TM} GFX^{TM} microspin column and DNA bound to the membrane. A wash buffer was applied and the membrane bound DNA was washed to remove any salts or contaminants and DNA dried. The purified DNA was then eluted from the column using sterile nuclease-free water and stored at -20^{o}C for subsequent use. (N.B. It was found that due to the formation of strong secondary structures, the H- and K-ras DNA given could not be used in a PCR reaction despite substantial optimisation attempts. For these reasons, the pDONR vectors were purchased from Addgene, as stated).

2.2.3 Construction of entry clones

To facilitate the transfer of the PCR product (*att*B sequence flanked N-ras DNA) into an *att*P containing donor vector (pDONR^{TM}207 vector), resulting in the formation of an entry clone, a Gateway^{TM} BP recombination reaction was performed. This was in accordance with the manufacturers instructions and summarised in **figure 2.1**. The BP reaction mix, containing the PCR product, donor vector and BP clonase, was incubated at 25^{o}C for 1 hour. After this

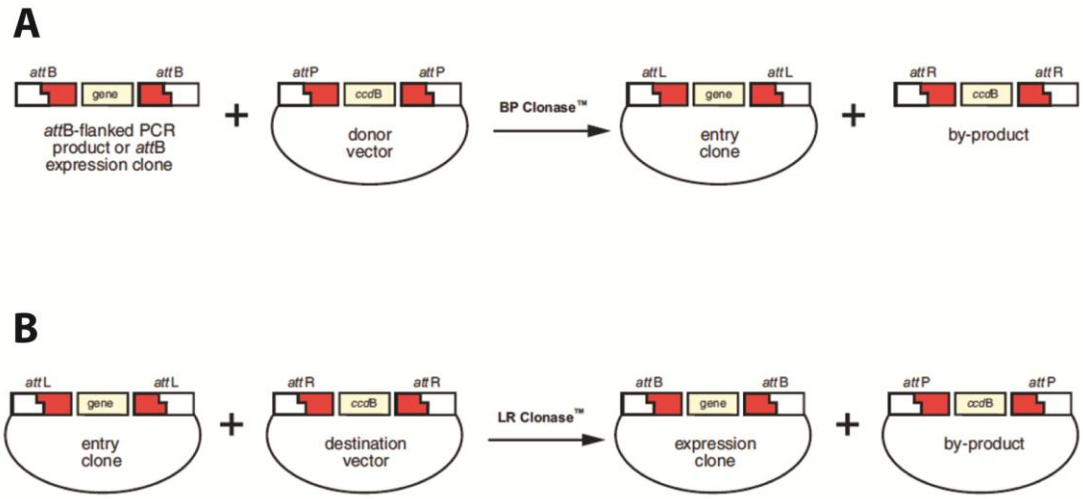


Figure 2.1: Gateway™ Technology BP and LR reactions. (A) Diagramatric summary of BP reaction for production of entry clone. (B) Summary of the LR recombination reaction, which is carried out in order to create an expression clone containing the gene of interest.

Image is from Gateway™ Technology Manual (Invitrogen, UK).

incubation, proteinase K was added, the reaction mix incubated at 37^{o}C for 10 minutes, which terminated the reaction. This reaction mix was then transformed into competent *E. coli* and entry clones selected for using the appropriate antibiotic (10µg/ml gentamycin for N-ras and 100µg/ml spectinomycin for purchased H- and K-ras). Bacteria were plated out onto LB-agar supplemented with the appropriate antibiotic and incubated overnight at 37^{o}C. Individual colonies were subsequently selected, purified and sequenced (by Eurofins MWG Operon) before continuing to LR reaction for production of expression clones.

2.2.4 Construction of expression clones

The Gateway^{TM} LR recombination reaction was used to transfer the gene of interest into an *att*R-containing destination vector to create an *att*B-containing expression clone. This reaction was used to generate GST, GFP and Myc-tagged expression clones using pDEST^{TM}15, pEGFP-C1 and pDEST^{TM}Myc respectively. This reaction was carried out in accordance with the manufacturers instructions and summarised in **figure 2.1**. The purified entry clone was incubated with the chosen destination vector and the LR Clonase^{TM} reaction buffer at 25^{o}C for 1 hour. The subsequent addition of proteinase K and incubation for 10 minutes at 37^{o}C terminated the reaction. This reaction mix was then used to transform chemically competent *E. coli*, the expression clones selected for using the appropriate antibiotic and the plasmid DNA isolated and purified. The DNA obtained was sequenced by Eurofins MWG Operon prior to any subsequent use in further assays.

2.2.5 Site Directed Mutagenesis

Site directed mutagenesis was used to construct two point mutants of full length PAK4 (P105,108A and P287,289A) and a PAK4 RNAi rescue construct (see **Table 2.3** for primer sequences).

The PCR reactions were performed using the Stratagene QuikChange® Site-Directed Mutagenesis kit which uses *PfuTurbo*® DNA polymerase and a temperature cycler. The reaction was set up in a 0.2ml PCR tube as follows: 5µl of 10x reaction buffer (100mM KCl, 100mM $(NH_4)_2SO_4$, 200mM Tris-HCl pH 8.8, 20mM $MgSO_4$, 1% Triton® X-100, 1mg/ml nuclease-free BSA), 50ng of template DNA (PAK4 entry clone), 1µl dNTP mix and 125ng of mutagenic forward and reverse primers. The total reaction volume was made up to 50µl with ddH_2O. To this 1µl *PfuTurbo*® DNA polymerase (2.5U/µl) was added. The specific thermo cycling parameters are described in **table 2.7**.

Number of cycles	Temperature	Time
1 Cycle	95°C	30 seconds
18 Cycles	95°C 55°C 68°C	30 seconds 1 minute 8 minutes*

Table 2.7: Cycling Parameters for the QuikChange Site-Directed Mutagenesis method.

*1 minute/kb of plasmid length.

Following the temperature cycling 1µl of the *Dpn* I restriction enzyme (10U/µl) was added to the amplification reaction in order to digest the parental (non mutated) supercoiled dsDNA template and select for the newly synthesized DNA containing the mutations. The reaction mixture was mixed by pipetting up and down several times, spun down in a microcentrifuge for one minute and then incubated at 37^{o}C for precisely one hour. Following this, 5µl of the reaction mixture was transformed into chemically competent *E. coli* bacteria.

2.2.6 Transformation of *Escherichia coli* cells

The heat shock method was used to transform *E. coli* bacteria, the strain used varied between different assays. Vials of bacteria, which were stored at -80^{o}C, were thawed for 10 minutes on ice prior to transformation. Typically, 5µl of plasmid DNA was added to the bacteria and mixed by tapping. These cells were then incubated of ice for 30 minutes. Following this, bacteria were heat shocked by incubating the vials containing the reaction mix at 42^{o}C. The length of heat shock varied slightly between *E. coli* strains (see **table 2.8** for variations in the procedures). The reaction mix was then placed on ice for 5 minutes. A volume of L-broth was then added to the reaction mix (see **table 2.8**) and then vials were incubated at 37^{o}C in a shaking incubator for one hour at approximately 225rpm.

Bacteria	Length of Heat Shock	Time on Ice after heat shock	Volume of L-broth added
One Shot® TOP10	30 seconds	2 minutes	250µl
BL21-A1™ One Shot®	30 seconds	2minutes	250µl

DH5α™	20 seconds	2 minutes	950µl
NEB 10-beta	30 seconds	5 minutes	950µl
Lemo21 (DE3)	10 seconds	5 minutes	950µl

Table 2.8: Transformation procedure of chemically competent *E. coli* bacteria

Subsequently, 200-300µl of the transformation reaction was plated onto pre-warmed LB plates containing the appropriate antibiotic for plasmid selection. The plates were inverted and incubated at 37°C overnight. The following day, colonies were picked and grown in L-broth containing the appropriate selection antibiotic and either grown up for use in various procedures or made into a glycerol stock.

2.2.7 DNA Preparation

Plasmid DNA was isolated and purified from *E.coli* bacteria using either the Invitrogen Purelink™ HiPure plasmid DNA purification maxiprep kit, the QIAfilter™ Plasmid Midi-prep kit or the Purelink™ HiPure Plasmid Filter Mini-prep kit, dependent on the DNA yield required. L-broth, with the appropriate selection antibiotic added, was inoculated with the transformed bacteria and grown overnight at 37°C in a shaking incubator. DNA was purified as per the manufacturers protocol and resulting DNA resuspended in TE buffer and stored at -20°C for future use.

2.3 Methods - Cell Biology

2.3.1 Cell Lines & Culture Conditions

Several difference cancerous and non-cancerous pancreatic cell lines were used and described here. Two epithelial cell lines included: HPDE cells which are a human papillomavirus (HPV)-16 E6E7 immortalised cell line, which were derived from normal adult pancreatic tissue (Furukawa et al., 1996) and dechTERT cells, which are primary cells collected and hTERT immortalised previously (Li et al., 2009) but have not been widely used. Three cancer cell lines were also used, including; Capan1, a well differentiated, colony forming cell line which was sourced from a liver metastasis, with mutations is *KRAS, TP53, INK4A, SMAD4* and *BRCA2* (Deer et al., 2010; Sipos et al., 2003). Also used, was another colony forming cell line, PaTu8988S that was isolated from a liver metastasis of a primary pancreatic adenocarcinoma together with PaTu8988T, which is a poorly differentiated, highly invasive cell line. These carry mutations in *KRAS* and *TP53* with methylation of the 5' CpG island of *INK4A* (Dammann et al., 2003; Elsasser et al., 1992). For use in the organotypic model of pancreatic cancer, PS-1 cells which were immortalised using ectopic human telomerase reverse transcriptase (hTERT) by members of Dr Hemant Kocher's laboratory (Froeling et al., 2009; Li et al., 2009). The strain was verified as being of stellate origin based on the expression of characteristic stellate cell markers. These included presence of lipid droplets in the cytoplasm and expression of various cytoskeletal proteins such as GFAP, desmin, vimentin and αSMA. They have been shown to grow well in culture, without the loss of phenotypic characteristics during continuous passage.

All cell lines were cultured as adherent monolayers in sterile tissue culture flasks. They were incubated in a humidified atmosphere of 5% CO_2 at 37^{o}C and maintained with regular media change. PaTu8988S and PaTu8988T (from Dr. Frank Ulrich Weiß, Department of Medicine, Ernst Moritz Arndt Universität Greifswald, Greifswald, Germany), HEK293 (ATCC) and DechTERT (from Mr Hemant Kocher, Barts Cancer Institute, UK) cells were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% v/v fetal bovine serum (FBS) and 1mM penicillin/streptomycin. Capan1 (from Mr Hemant Kocher, Barts Cancer Institute, UK) cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) media supplemented with 10% v/v FBS with 1mM penicillin/streptomycin. HPDE cells (from Mr Hemant Kocher, Barts Cancer Institute, UK) were maintained in keratinocyte serum-free media (K-SFM) supplemented with 5ng/ml epidermal growth factor (EGF), 0.1mg/ml bovine pituitary extract (BPE) and 1mM penicillin/streptomycin. PS-1 cells (from Mr Hemant Kocher, Barts Cancer Insitute, UK) were maintained in DMEM:F12, supplemented with 10% v/v FBS, 1mM penicillin/streptomycin and 1µg/ml puromycin as a selection agent. To passage, cells were first washed with sterile phosphate buffered saline (PBS) and trypsinised with 0.5% Trypsin/Ethylenediaminetetraacetic acid (EDTA).

2.3.2 Calcium Phosphate Transfection

The calcium phosphate transfection kit was used to transfect HEK293 cells. Cells were seeded at a density of 1x10^{5}/ml and incubated at 37^{o}C overnight. On the day of transfection, 3-4 hours prior to the addition of the reaction mix,

the media was aspirated and replaced with fresh, warmed media. The reaction mix was set up using the conditions described in **table 2.9**.

	2cm tissue culture plate (2ml total volume)	10cm tissue culture plate (10ml total volume)
Tube A:	7.2µl 2M $CaCl_2$ 4µg DNA Make up to 60µl with sterile water	30µl 2M $CaCl_2$ 20µg DNA Make up to 300µl with sterile water
Tube B:	60µl 2x Hepes buffered saline	300µl 2x Hepes buffered saline

Table 2.9: Calcium phosphate transfection reaction mix.

The contents of tube A ($CaCl_2$, DNA and water mix) was added 100µl at a time, dropwise, to the 2x HBS in tube B, with aeration. This transfection mixture was then incubated at room temperature for 30 minutes, before being added dropwise to the cells and dispersed.

2.3.3 X-treme Gene Transfection

Cells were plated at the optimal seeding density (depending on application) of either $2x10^4$/ml or $1x10^5$/ml 24 hours prior to transfection in full growth media (10% FBS). On the day of transfection, the media was aspirated from the cells, which where then washed once with sterile PBS before the media was replaced with serum free OptiMEM media, free from antibiotics. The transfection mixture was then made up in OptiMEM serum free media, details of which are outline in **table 2.10**. To the OptiMEM the XtremeGENE HP transfection reagent and DNA were added at a ratio of 6:2 and incubated at room temperature for 30 minutes before being added dropwise to the cells

and dispersed. Cells were then returned to the incubator and maintained at 37^{o}C for 6 hours, after which the OptiMEM was aspirated and replaced with pre-warmed, full growth media.

Culture Vessel	Total Volume of Media	Reaction Mix total Volume	DNA	Volume of XtremeGENE
2cm dish	2 ml	100 µl	2 µg	6 µl
10cm dish	10 ml	500 µl	10 µg	30 µl

Table 2.10: X-treme Gene transfection reaction mix.

2.3.4 Hiperfect siRNA transfection

Cells were plated at an appropriate density (2x10^{4}/ml) the day before transfection in full growth media (10% FBS). The next day, the reaction mix was set up as follows: 60nM of siRNA (**table 2.11**) was diluted in 100µl serum-free OptiMEM media. Equal concentrations of a control siRNA were used in each experiment. To the reaction mix, 12µl of HiPerfect was added. (Note: these volumes were for cells seeded in a 6 well plate in a total volume of 2ml; volumes were scaled up accordingly for larger experiments). This was then incubated at room temperature for 30 minutes before being added dropwise to the cells. Cells were then incubated at 37^{o}C for various time frames depending on the assay. For longer periods of time i.e. in excess of 72 hours, cells were trypsinised and reseeded to avoid becoming over-confluent.

Oligo Name	Target Sequence	Company
Control siRNA	AATTCTCCGAACGTGTCACGT	Qiagen
PAK4 siRNA1	GGTGAACATGTATGAGTGT	Ambion
PAK4 siRNA2	CGAGAATGTGGTGGAGATGTA	Qiagen
PAK4 siRNA3	GGATAATGGTGATTGGAT	Thermo Scientific
PAK4 siRNA5	GGGTGAAGCTGTCAGACTT	Thermo Scientific
Gab1 SMARTpool siRNA	GAGAGTGGATTATGTTGTT GATGCTGGATTGACATTTA CATCAAAGCTAGACACTAT ATACTTAGATCTCGACTTA	Thermo Scientific

Table 2.11: Sequences of siRNAs used.

2.3.5 Collagen Coating

Type I rat tail collagen was diluted to 50µg/ml in 0.02M glacial acetic acid, which had been filter sterilized. The dilute collagen solution was added and left on a level surface at room temperature for 60 minutes. The collagen was then aspirated and washed twice with sterile PBS before cells were seeded as usual.

2.3.6 Time-lapse Microscopy

Cells were seeded onto collagen coated wells of a 6 well plate at a density of $2x10^4$/ml. Where siRNA was used prior to movie acquisition cells were plated onto plastic, treated with the siRNA and then trypsinised and replated onto collagen coated plated on the day of the time-lapse. Immediately prior to time-lapse acquisition, the media was supplemented with 20mM Hepes before plates were sealed with parafilm and placed on the automated and

heated stage of an Olympus IX71 inverted time-lapse microscope. Images were collected using a Retiga SRV CCD camera with images being captured at 5-minute intervals for a total time period of 12 hours using Image-Pro Plus software.

Following acquisition the movie files were extracted and saved as AVI files. These were then imported into the ImageJ software programme and cells could be manually tracked using the manual tracking plugin, MTrackJ. This resulted in the generation of a sequence of position coordinates, which could then be imported into the chemotaxis and migration tool from Ibidi. This allowed further analysis; the generation of migration plot profiles and the mean velocity and distance values to be calculated. In general, approximately 60-100 cells were tracked over at least three independent experiments per condition.

2.3.7 Inhibitors

LY294002 (molecular formula: $C_{19}H_{17}N_3$) is known to be a highly selective inhibitor of PI3K and has been shown to block PI3K-dependent AKT phosphorylation and kinase activity. A stock solution, at a concentration of 10mM, was made by diluting the lyophilized powder in DMSO. Aliquots were stored at -20^{o}C until required. For use in all assays the stock solution was diluted further in culture medium to establish a working concentration of 20nM. An equal volume of DMSO was used as a control in all experimental procedures.

2.3.8.MTT Assay

Prior to performing the MTT assay, PaTu8988T cells were treated with siRNA to transiently knockdown PAK4. A non-targeting siRNA was also used as a control. A 24 well sterile tissue culture plate was coated with collagen as described in **section 2.3.5**. Cells were seeded at a density of $5x10^3$ and then incubated for 72 hours. The MTT powder was made up to a working concentration of 500µg/ml in DMEM media, fresh before each experiment. Culture media was carefully aspirated from each well and cells washed twice with sterile PBS before 1ml MTT:DMEM solution was applied. Cells were incubated in the presence of the MTT at 37^{o}C for 3 hours. After this incubation, the MTT:DMEM was carefully removed and 500µl DMSO was used to solubilize the converted dye. This was pipetted up and down several times and then incubated at 37^{o}C for a further 10 minutes. Each sample was then mixed again by pipetting and transferred to an Eppendorf tube. Subsequently, the absorbance was measured at a wavelength of 562nm using a Nanodrop.

2.3.9 HGF Stimulations

Cells were seeded at densities appropriate for the subsequent assay and subsequently maintained at 37^{o}C in a humidified atmosphere with 5% CO_2. The night before the HGF stimulation, the culture media was removed and cells were washed twice in sterile PBS. They were then maintained in media containing 0% FBS (starve media). The next day for lysates HGF was added at various time points at a concentration of 10ng/ml before cells were lysed and subsequently stored at -20^{o}C. For use in migration assay, HGF was

added immediately prior to commencing time-lapse microscopy, also at a concentration of 10ng/ml.

2.3.10 Immunofluorescence

Cells cultured on coverslips.

When seeding cells on coverslips, these were sterilized prior to use. Coverslips were incubated overnight at room temperature in a solution of 40% 1M hydrochloric acid and 60% ethanol (96% solution). The coverslips were then rinsed three times in distilled water and boiled, rinsed a further six times in distilled water and then autoclaved.

Cells were seeded onto collagen-coated coverslips and left to settle overnight. They were then washed twice in PBS and fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Cells were then washed three times in PBS and permeabilised in 0.2% TritonX-100 for 5 minutes at room temperature, washed a further three times in PBS and then blocked with 3% BSA for 30 minutes. The coverslips were then incubated in primary antibody, diluted in 3% BSA for 2 hours at room temperature in the dark, washed three times in PBS and incubated with the appropriate secondary antibody for one hour at room temperature in the dark. To visualize the nucleus, cells were also incubated with DAPI diluted in 3% BSA for 10 minutes before a final three washed in PBS and one with distilled water. Coverslips were then mounted onto microscope slides using FluorSave, left at room temperature in the dark overnight and stored at -20^{o}C until being imaged. Cells were visualised using an Olympus IX71 inverted

microscope, using a 40X lens, and images analysed using ImageJ software.

Organotypic Culture Models

For paraffin embedded samples, the sections were de-waxed and rehydrated prior to staining. Slides were de-waxed using xylene twice for 5 minutes each time. To rehydrate the sections an alcohol gradient was used. Each wash was incubated at room temperature for 2 minutes as follows:

2 x 100% Ethanol

1 x 90% Ethanol

1 x 70% Ethanol

1 x 50% Ethanol

1 x double distilled water

Samples were then soaked in PBS (with calcium and magnesium). Antigen retrieval was then completed in order to break the protein cross-links, which are formed through formalin fixation. To do this, 0.01M tri-sodium citrate (dihydrate) at pH6 was brought to the boil. The slides were then immersed in the buffer and boiled for 10 minutes.

Following antigen retrieval slides were dried quickly and sections outlined with a wax pen, being careful not to touch the sections. The sections were then permeabilised through incubation in 0.2% TritonX-100 in PBS for 5 minutes at room temperature. Sections were washed twice in PBS and then quenched in a 1mg/ml sodium borohydride/PBS solution for 10 minutes.

Sections were then washed twice in PBS before being blocked for 30 minutes at room temperature. A blocking solution containing 2% BSA, 0.02% fish skin gelatin and 10% FBS was used. Sections were then incubated overnight at 4^{o}C in the dark with the appropriate primary antibody. The following day, sections were washed three times with PBS before being incubated for 1 hour at room temperature in the dark with the appropriate secondary antibody, followed by 10 minutes with DAPI. The sections were washed another three times in PBS and twice in dd$H_2$0. A small amount of fluorsave mounting medium was then pipetted onto a square coverslip. This was inverted onto the sections and gently pressed down to remove any air bubbles. The slides were then kept in the dark at room temperature overnight in order for the fluor save to set. Slides were stored at -20^{o}C until imaged. Images were collected on a Carl Zeiss LSM510 META laser scanning confocal microscope or a Nikon Eclipse Ti-E inverted A1R Si confocal microscope.

2.3.11 Organotypic Culture Models

On day one, the organotypic gel matrix was prepared. First, a cell suspension of PS-1 cells was made to get a final concentration of 2.5x10^{5} cells in a volume of 100ul per gel. The gels were made up in the ratios in **table 2.12**. Prior to use, all components were filter sterilized through a 20-micron syringe-top filter to remove any precipitate and were maintained on ice during the preparation to avoid polymerisation of the collagen and Matrigel^{TM}.

The gel was mixed thoroughly with care not to introduce any air bubbles. If yellow, the gel was neutralized through the addition of filter sterilized 0.1M sodium hydroxide (NaOH) until pink. Once mixed, 1ml was added dropwise to each well of a 24-well tissue culture plate, being careful not to introduce any air bubbles. The plate was then placed in an incubator for 1 hour at 37^{o}C until the gel had polymerized. After this, 1ml of DMEM with 10% FBS was added on top of each gel before being returned to the incubator and left overnight.

Component	Quantity
Collagen:Matrigel^{TM} (1:1)	7 volumes (3.5:3.5)
10X DMEM	1 volume
FCS	1 volume
Fibroblast suspension	1 volume

Table 2.12: Organotypic model matrix preparation

On day 2, a cell suspension was prepared containing 5x10^{5} PaTu8988T cells mixed with 2.5x10^{5} PS-1 cells per gel in 1ml DMEM containing 10% FBS. In order to study the effect of PAK4 knockdown on pancreatic cancer cell invasion, PaTu8988T cells had previously been treated with siRNA and to look at the effect of the PI3K inhibitor (LY294002) cells were seeded in the presence of the inhibitor. Once the cell suspension was prepared, the media was aspirated carefully from on top of each gel and 1ml of cell suspension then added dropwise to the centre of the gel. The gels were incubated at room temperature for 5 minutes to allow the cells to settle before being

returned to the incubator and maintained at 37^{o}C for 48 hours.

On day 3, the nylon sheets were prepared in the following manner. The required number of nylon sheets, which had previously been autoclaved to sterilize, were placed in a sterile petri dish. A collagen gel was then prepared (see **table 2.13**), with all components being maintained and mixed on ice.

Component	Quantity
Collagen	7 volumes
10X DMEM	1 volume
FBS	1 volume
DMEM containing 10% FBS	1 volume

Table 2.13: Components of collagen gel mixture

The gel was mixed carefully to prevent the introduction of air bubbles and neutralized with the addition of filter sterilized 0.1M NaOH until it was pink in colour. Once mixed, 250µl of the collagen mix was added dropwise to the centre of each sheet and the petri dish was incubated at 37^{o}C for 30 minutes to allow the collagen to polymerise. To crosslink the collagen, a 1% gluteraldehyde solution was prepared in sterile PBS. After 30 minutes, 10ml of the gluteraldehyde solution was added to the petri dish and then incubated at 4^{o}C for one hour. The nylon sheets were then washed three times in sterile PBS, once in DMEM containing 10% FBS and then left in 10ml DMEM with 10% FBS. The petri dish was sealed with parafilm and left at 4^{o}C overnight.

On day 4, the organotypic gels were raised onto steel grids to create an air-liquid interface. To do so, steel grids were placed into each well of a 6 well tissue culture plate. Onto each grid, a gel-coated nylon sheet was carefully placed. Then, using a sterile spatula, each gel was carefully removed from the 24 well culture plate and placed on top of the nylon sheet. The well of the plate was then filled until it reached the undersurface of the grid. This was then classed as day 1 of the organotypic invasion assay. The gels were then returned to the incubator and maintained at 37^{o}C. The culture media was replaced every two days and the gels were harvested after 14 days of culture, with day one being counted as the day gels were raised onto grids. A diagrammatic summary of the organotypic model set up is displayed in **figure 2.2**.

After 14 days, the gels were harvested by removing the whole gel and the nylon membrane from the grid. Gels were then carefully cut in half and placed in a universal tube. Gels were fixed in formal saline for 24 hours. The formal saline was then replaced with 70% ethanol and then maintained at 4^{o}C and sent for processing (paraffin embedding and sectioning) at the Pathology laboratory at St Thomas Hospital, London. Sectioned samples were then stained (as described in **section 2.3.10**) and analysed (as described in **section 2.5.1** and **figure 2.3**).

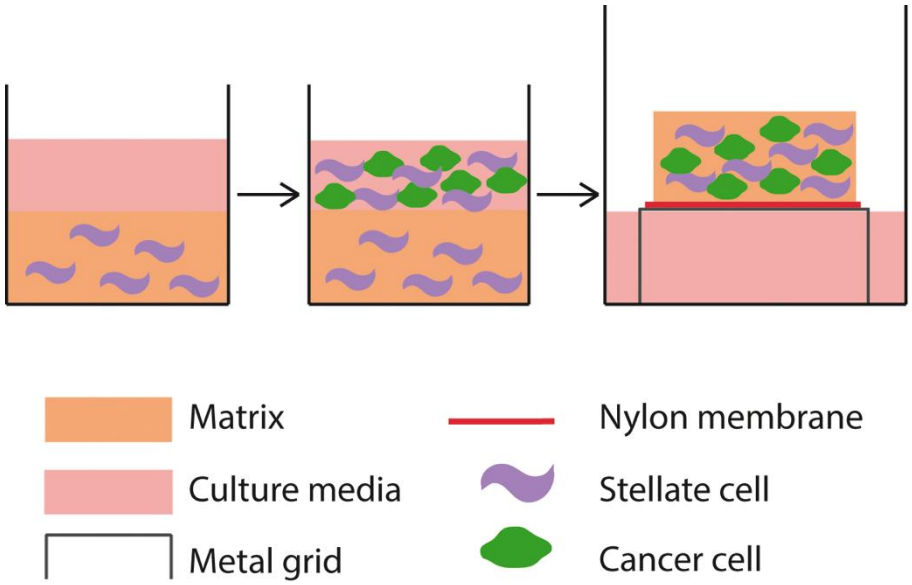


Figure 2.2: Organotypic model set up. A collagen:matrigel^{TM} matrix was produced containing pancreatic stellate cells. The next day a mixture of pancreatic cancer cells and stellate cells (ratio 2:1) were seeded on top. 48 hours later, the matrix was raised onto a collagen-covered nylon membrane on top of a steel grid. Culture media was used to create an air-liquid interface to promote invasion of cells down into the matrix. Culture media was replaced every two days for a total of fourteen days. Gels were then formalin fixed overnight, maintained in ice-cold ethanol and sent for processing.

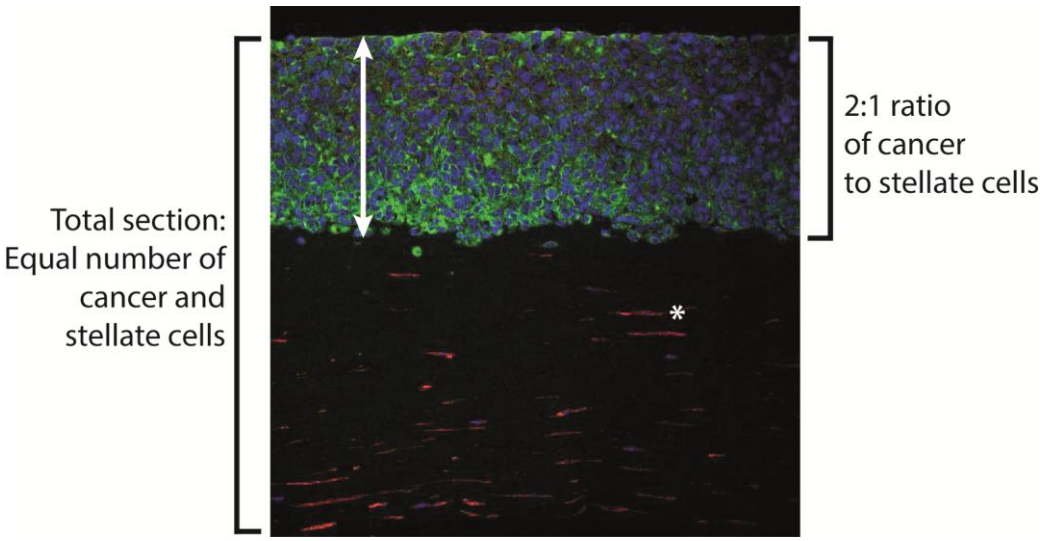


Figure 2.3: Organotypic model analysis. After the organotypic gels had been processed and sectioned, they were stained as above for DAPI (blue), pan-cytokeratin (green) and SMA (red). For analysis of invasion depth, three randomly selected points were measured using ImageJ. This is indicated by the white arrow. A total number of at least 30 measurements were taken per condition over three independent experiments. Any obvious areas of artifactual invasion (i.e. at the edges of gel sections or through processing) were excluded. * Indicates PS-1 cell stained for SMA, which was pre-mixed into the matrix before a 2:1 ratio of cancer (PaTu8988T): stellate (PS-1) cells were seeded on top. Therefore total number of cells per gel is equal.

2.4 Methods - Biochemistry

2.4.1 Protein Preparations

For the purification of GST-fusion proteins, *E. coli* BL21-A1 cells were transformed with GST expression plasmids. The transformed bacteria were cultured in 5ml L-broth to which 100µg/ml of the antibiotic ampicillin was added. These cultures were incubated overnight at 37^{o}C. The next day 2ml of this culture was added to 200ml of L-broth inoculated with ampicillin, forming a 1 in 200 dilution. These were incubated at 37^{o}C for approximately 2 hours until the optical density reached 0.6. In order to induce the bacterial cultures, 2ml of a 20% L-Arabinose solution was added before the cultures were incubated overnight at 20^{o}C. The following day, the cultures were harvested by centrifuging at 6000 rpm for 15 minutes. The supernatant was discarded and the pelleted bacteria were resuspended in 15ml PBS containing protease inhibitor cocktail. To disrupt the cells, the bacteria were subjected to sonication for 3 minutes with a pulse every second and at a 70% amplitude. Cell debris was removed by centrifugation for 10 minutes at 4800 rpm and the supernatant retained. During this time, GST beads were pre-washed using the PBS plus inhibitor cocktail solution three times. Beads were then added to the supernatant before incubation for 2 hours at 4^{o}C on a rotating wheel. Following this incubation period, the beads were pelleted by centrifugation at 500 x gravity for 5 minutes. Beads were washed three times in the PBS plus protease inhibitor cocktail solution before a freeze down buffer was added in equal measure. These were then stored at -80^{o}C until use.

2.4.2 GST Pulldown Assay

HEK293 cells were used within these assays and plated at a density of $1x10^5$/ml into 10cm tissue culture plates and incubated overnight at 37^oC. The next day the cells were transfected with the appropriate GFP-tagged construct using the calcium phosphate transfection kit (see **section 2.3.2**). After 24 hours cells were lysed using an optimized pulldown lysis buffer (see section **2.1.2 Buffers**). GST beads, which had been pre-washed in the lysis buffer, were used to pre-clear the lysates. These were incubated for one hour at 4^oC on a rotating wheel. Lysates were centrifuged at 2000 rpm for 2 minutes and the supernatant transferred to a fresh eppendorf tube. GST purified protein beads, which had also been pre-washed in the lysis buffer, were then added to the lysates which were incubated for a further two hours at 4^oC on a rotating wheel. Subsequently the lysates were centrifuged at 2000rpm for 2 minutes and the unbound fractions were discarded. The beads were then washed three times using pulldown wash buffer and 30µl of 2x SDS gel sample buffer added. Samples were then boiled for 3 minutes and stored at -20^oC until used in immunoblotting.

For each experiment a GST alone expression plasmid was used as a control and approximately equal amounts of GST alone and GST fusion proteins were used in each assay. For plasmids see **table 2.2**.

2.4.3 Western Blotting

Isolation of Protein

When cells were approximately 70% confluent, they were washed twice in

PBS and lysed for 10 minutes on ice in 0.5% NP-40 lysis buffer supplemented with 1mM DTT, 1µg/ml aprotinin, 10µg/ml leupeptin, 1mM PMSF, 50mM sodium fluoride and 1mM sodium orthovanadate. Cells were scraped and transferred to eppendorf tubes. Cell debris was collected by centrifuging the lysates at 13,000 x g for 10 minutes at 4^{o}C. The supernatant was then collected and transferred to a fresh eppendorf and samples prepared through the addition of a 6 X Laemmli buffer (diluted to 1 X) before samples were boiled for 3 minutes. Whole cell lysate samples were then subsequently stored at -20^{o}C until use.

Gel electrophoresis and immunoblotting

Lysates were boiled for 3 minutes prior to use. An equal amount of denatured, cellular proteins were then separated on 7.5-12.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins were then electroblotted onto nitrocellulose membrane at 70-100 volts for 1-2 hours (dependent on the percentage gels used). Non-specific binding was blocked through incubation of the membrane in 5% skimmed milk in 0.1% TBS-Tween20 or 5% BSA in 0.1% TBS-Tween20, followed by overnight incubation in the primary antibody at 4^{o}C (see **table 2.4**). Membranes were then washed three times, for 10 minutes, in 0.1% TBS-Tween20 prior to incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (see **table 2.5**) for one hour at room temperature. Membranes were then washed a further three times, for 10 minutes, in 0.1% TBS-Tween20. Specific protein bands were then visualised using an ECL chemiluminescence detection kit.

Stripping of Nitrocellulose Membranes

If membranes were to be reprobed, they were incubated in stripping buffer twice for 15 minutes each (at room temperature for mild stripping or at 50^{o}C if a harsh stripping buffer was used) with constant agitation. Membranes were then washed twice for 10 minutes in PBS and twice for 10 minutes in TBS-Tween20 before being blocked and probed with antibody as previously stated.

2.5 Data & Statistical Analysis

2.5.1 Organotypic model

For organotypic cultures, three gels from three independent experiments were analysed and several randomly selected areas imaged by an independent observer. The invasion depth was measured at three points in each sample using ImageJ. The areas of invading cells were calculated after exclusion of non-uniform invasion at the edges of the gel. Any areas where obvious artifactual invasion had occurred in identifiable areas (that could be due to processing) were also omitted.

2.5.2 Densitometry

For western blotting, densitometric analysis of specific bands was performed using ImageJ software. In order to obtain a semi-quantitative measurement of the level of total protein, all band densities were normalised to a loading control. In all circumstances, GAPDH was used as a loading control and was detected on the same membrane as the protein of interest.

2.5.3 Statistics

Where appropriate, results were subjected to statistical analysis using unpaired student t-tests. Values were considered statistically significant if the P-value was <0.05. Error bars represent SEM.

Chapter 3
Results: Part 1
Characterisation of pancreatic cell lines

Chapter 3: Characterisation of pancreatic cell lines

3.1 Introduction

This project aimed to understand the role of PAK4 in pancreatic cancer, so it was first necessary to characterise a range of cell lines. Five cell lines were chosen for initial investigation. This included two epithelial cell lines; HPDE cells which are a human papillomavirus (HPV)-16 E6E7 immortalised cell line which were derived from normal adult pancreatic tissue (Furukawa et al., 1996) and dechTERT cells, which were primary cells collected and hTERT immortalised previously (Li et al., 2009), but have not been widely used. Three cancer cell lines were also selected; Capan1, a well differentiated, colony forming cell line which was sourced from a liver metastasis, with mutations is KRAS, TP53, INK4A, SMAD4 and BRCA2 (Deer et al., 2010; Sipos et al., 2003). Another colony forming cell line, PaTu8988S, isolated from a liver metastasis of a primary pancreatic adenocarcinoma together with PaTu8988T, which is a poorly differentiated, highly invasive cell line. These sister cell lines carry mutations in KRAS and TP53 with methylation of the 5' CpG island of INK4A (Dammann et al., 2003; Elsasser et al., 1992).

It has been well established that the PI3K pathway is upregulated in pancreatic cancer (Eser et al., 2013) and contributes to disease development and progression. It is also known that this pathway can be stimulated either through c-Met downstream of HGF or through Ras (Ebi et al., 2011). c-Met is known to have increased expression within pancreatic cancer (Herreros-Villanueva et al., 2012; Li et al., 2011a) and mutations in K-ras are found in more than 90% of pancreatic cancers (Collins et al., 2012). It was therefore

considered important to determine the differential expression profile for these and associated proteins within the cell lines being selected for characterisation.

Links between PAK4 and pancreatic cancer have also been demonstrated through some previous genomic studies (Chen et al., 2008; Kimmelman et al., 2008). The PAK proteins are a family of serine/threonine kinases, of which there are six isoforms; these are divided into two groups, based on structural similarities and sequence homology. Group I is made up of PAKs 1, 2 and 3 and group II contains PAKs 4, 5 and 6 (Bokoch, 2003; Jaffer and Chernoff, 2002). It is well documented that PAKs act as effectors for a wide range of signalling pathways and contribute to changes in cellular motility, morphogenic processes and cell polarity (Arias-Romero and Chernoff, 2008; Bagrodia and Cerione, 1999; Hofmann et al., 2004; Sells and Chernoff, 1997). As PAK proteins are involved in the regulation of many cellular processes, this infers that abnormalities in PAK functioning are likely to contribute to a number of disease states including cancer (Dummler et al., 2009; Kumar et al., 2006; Kumar and Vadlamudi, 2002). However, the complete expression profile of the PAK family kinases has not been well characterised within pancreatic cancer. Therefore, alongside the other proteins, the panel of cell lines was probed for PAK1-6 expression.

This first chapter shows the results from immunofluorescence and western blotting studies on a range of pancreatic cell lines. The results gathered throughout these initial characterisation studies were used to justify which

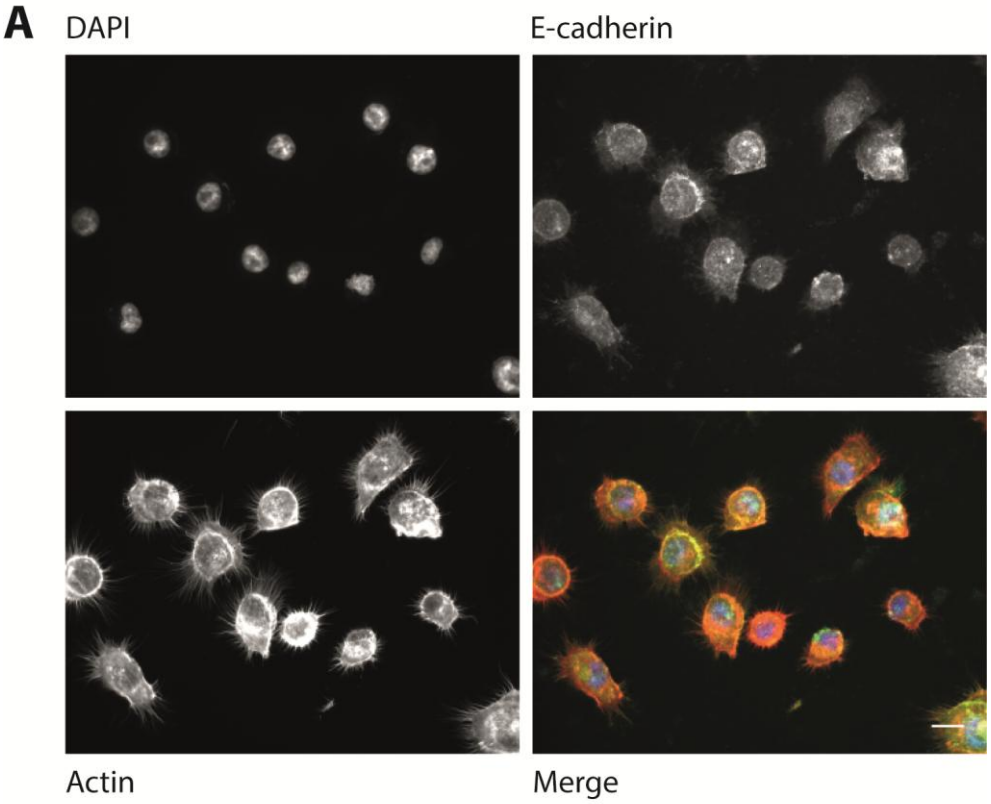
cell lines would be most appropriate for use throughout the course of this investigation.

3.2 Results

3.2.1 E-cadherin expression

Upon first culturing the cells, it was noted that although the HPDE cells were selected as they are representative of an epithelial phenotype, they appeared to lack the defined characteristics of epithelial cells. They remained as single cells, with no monolayer formation being observed and no evidence of cell:cell junctions. Thus it was decided to define the epithelial versus mesenchymal phenotype of the selected cell lines by testing E-cadherin localisation and expression.

To assess the epithelial characteristics of the selected cell lines, all of the cells were seeded onto coverslips and stained for E-cadherin. E-cadherin is a key component of intercellular junctions, with continued expression and functional activity a key requirement in the maintenance of stable junctions between cells (Gumbiner, 1996; Li and Mattingly, 2008). It was evident that Capan1 (**figure 3.1C**) and PaTu8988S (**figure 3.1D**) cells expressed E-cadherin, with the expression being concentrated at junctions between the cells. Although HPDE cells (**figure 3.1A**) expressed E-cadherin, there was no evidence of colony or monolayer formation evident, nor any junctional staining. E-cadherin mediated cell-cell adhesions are dependent on extracellular calcium (Gumbiner, 1996). This extracellular calcium serves to rigidify the extracellular domains of E-cadherin and enables homophilic interactions (Kim et al., 2011a; Nagar et al., 1996). Thus, HPDE were seeded in the presence of calcium nitrate (1g/L) as the addition of calcium to the media has been shown to increase extracellular calcium levels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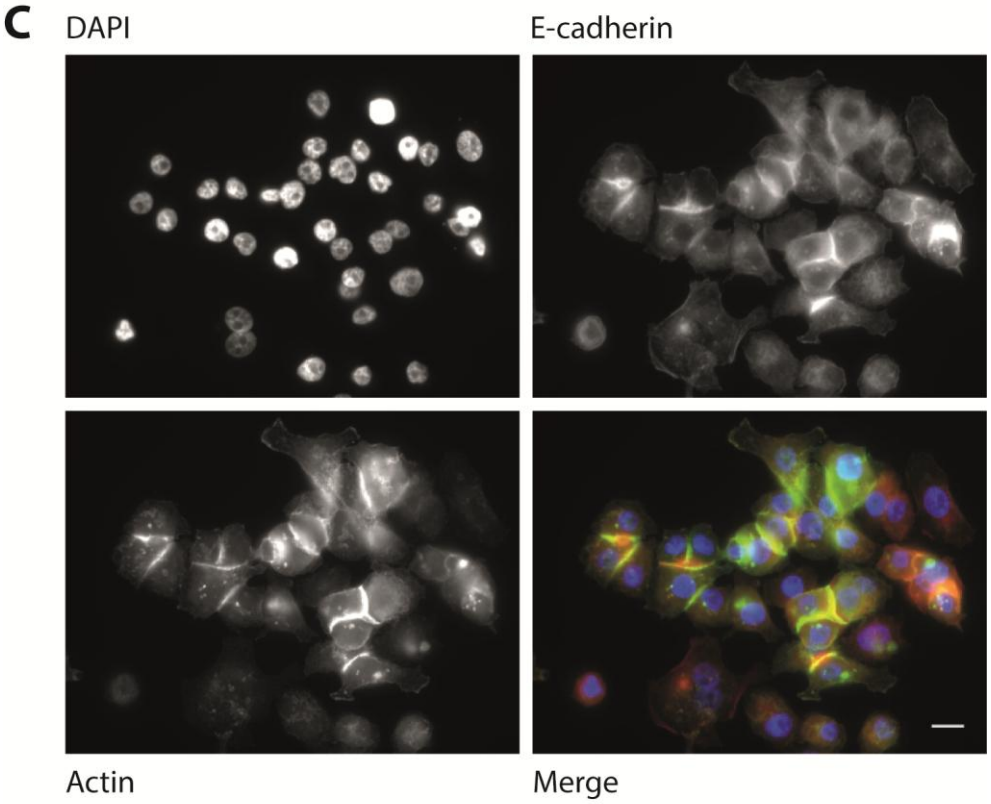
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Figure 3.1: E-cadherin expression in pancreatic cell lines. HPDE (A), dechTERT (B), Capan1 (C) and PaTu8988S (D) cell lines were subjected to immunoflourescent staining to observe the expression of E-cadherin (green), in combination with DAPI (blue) and actin (red). Both Capan1 and PaTu8988S cells express E-cadherin, which appears concentrated at the junctions forming between cells within colonies. DechTERT cells appear to express no E-cadherin despite this being a characteristic of epithelial cells. HPDE cells express E-cadherin, although do not form cell-cell contacts. Scale bar = 10μm.

and induce the formation of cell-cell interactions (Martin et al., 1991).

The HPDE cells responded well to the calcium nitrate treatment and displayed a more typical epithelial appearance; E-cadherin was observed at the junctions now formed between cells, as highlighted by the white arrow (**figure 3.2**). Unlike HPDE cells, the dechTERT cell line appeared to lack E-cadherin almost entirely (**figure 3.1B**). To compliment these immunofluorescence studies, western blotting was carried out to examine the expression of E-cadherin across the cell lines (**figure 3.3A**). Whole cell lysates from HPDE cells +/- calcium nitrate were produced, but little difference in protein expression was observed. It is known that the interaction between the cytoplasmic tail of E-cadherin with β-catenin is crucial for junctional stability, providing links to the actin cytoskeleton and further enhancing junction formation and strength (Conacci-Sorrell et al., 2002; Tian et al., 2011a). So, in conjunction with E-cadherin, β-catenin was also probed for (**figure 3.3A**) and the relative density quantified (**figure 3.3B**).

E-cadherin was expressed only in the two colony forming cancer cell lines and the HPDE cells. It was also confirmed that the dechTERT cells expressed no E-cadherin, yet had high levels of β-catenin expression. It has already been shown that PaTu8988S and PaTu8988T cells have differential β-catenin expression (Marques et al., 2009), with PaTu8988S cells having observably higher levels. Marques *et al* also demonstrated that the lack of E-cadherin expression in the PaTu8988T cells lead to accumulation of β-

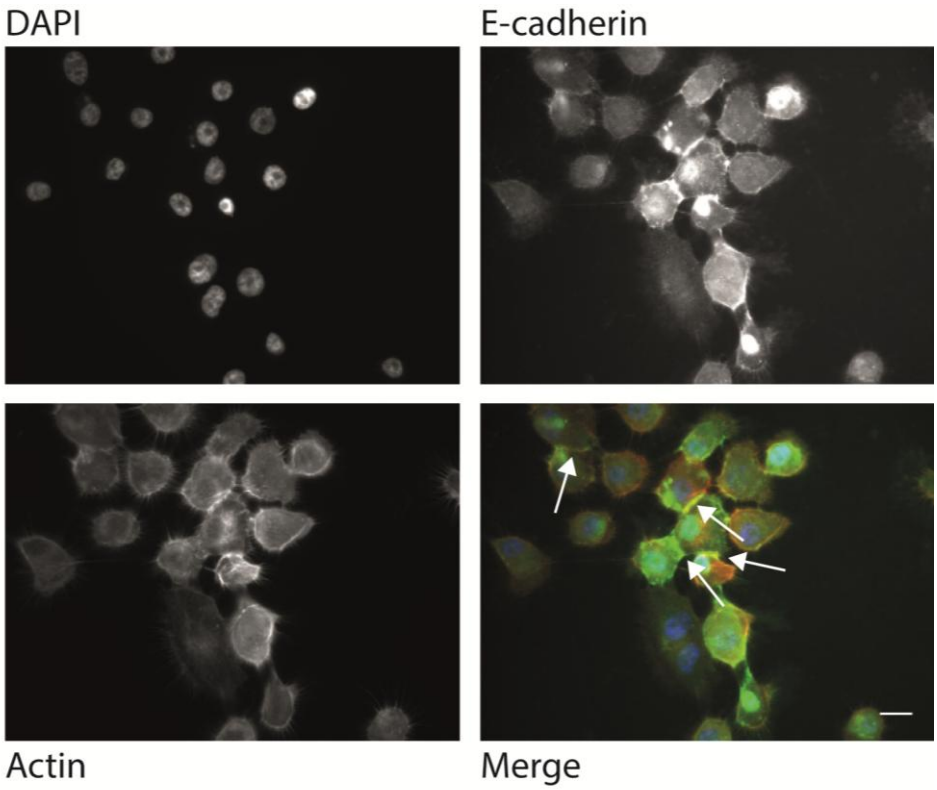


Figure 3.2: Calcium nitrate induces the formation of cell-cell contacts in HPDE cells. In order to make HPDE cells for epithelial in appearance and behavior, cells were seeded in the presence of calcium nitrate (1g/L). This forced cells to form points of contact. These cells were stained for E-cadherin (green), DAPI (blue) and actin (red) and it could be observed that in comparison to cells seeded without calcium that now E-cadherin was concentrated at junctions formed between cells, as indicated with the white arrow. Scale bar = 10μm.

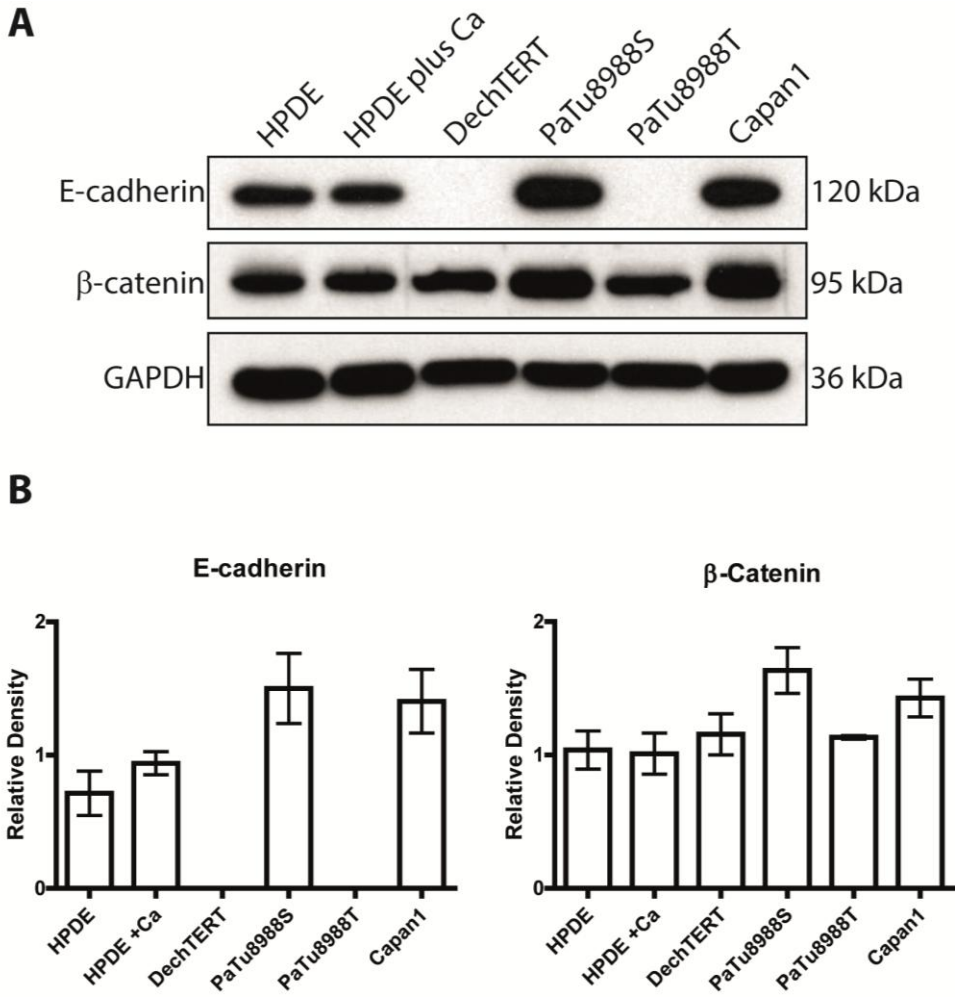


Figure 3.3: Expression of E-cadherin and β-catenin in pancreatic cell lines. (A) The expression of E-cadherin was confirmed by western blotting. In conjunction with this, the expression of β-catenin was also detected. (B) Relative expression was quantified via densitometry where the relative density/expression was first normalised to the loading control (GAPDH). Blots shown are representative of 3 independent experiments each of which was then subjected to densitometry (as described above) for quantificational purposes and shown in graphical form (B).

catenin in the cytoplasm, whereas PaTu8988S cells had colocalisation of β-catenin and E-cadherin at complexes located at the plasma membrane which is linked to the invasive potential of the cell lines. The studies carried out as part of this characterisation are therefore in line with this previous work (Marques et al., 2009).

3.2.2 Further characterisation of the cell line dechTERT

The dechTERT cell line was immortalised and characterised previously (Li et al., 2009). However, during the preliminary characterisation of all pancreatic cell lines, it was found that they lacked the classic epithelial marker E-cadherin (**figure 3.3**). Further characterisation was therefore carried out through immunofluorescent studies to further interrogate the nature of this cell line. The dechTERT cells were stained for a range of proteins including classical epithelial and tight junction proteins and markers of EMT (**figure 3.4**). The expression level of β-catenin had already been demonstrated (**figure 3.3A**) and so the dechTERT cells were stained for the presence and localisation. It was noted, that there was an accumulation of β-catenin in the perinuclear region. The levels of cytoplasmic β-catenin are usually tightly controlled (Olmeda et al., 2003); E-cadherin usually recruits β-catenin to the plasma membrane, leading to sequestration and preventing its translocation (Orsulic et al., 1999) and so, with the lack of E-cadherin observed an accumulation of β-catenin within the cytoplasm is expected.

A loss of E-cadherin is considered to be one of the main molecular events responsible for dysfunction of cell-cell adhesion and is usually associated

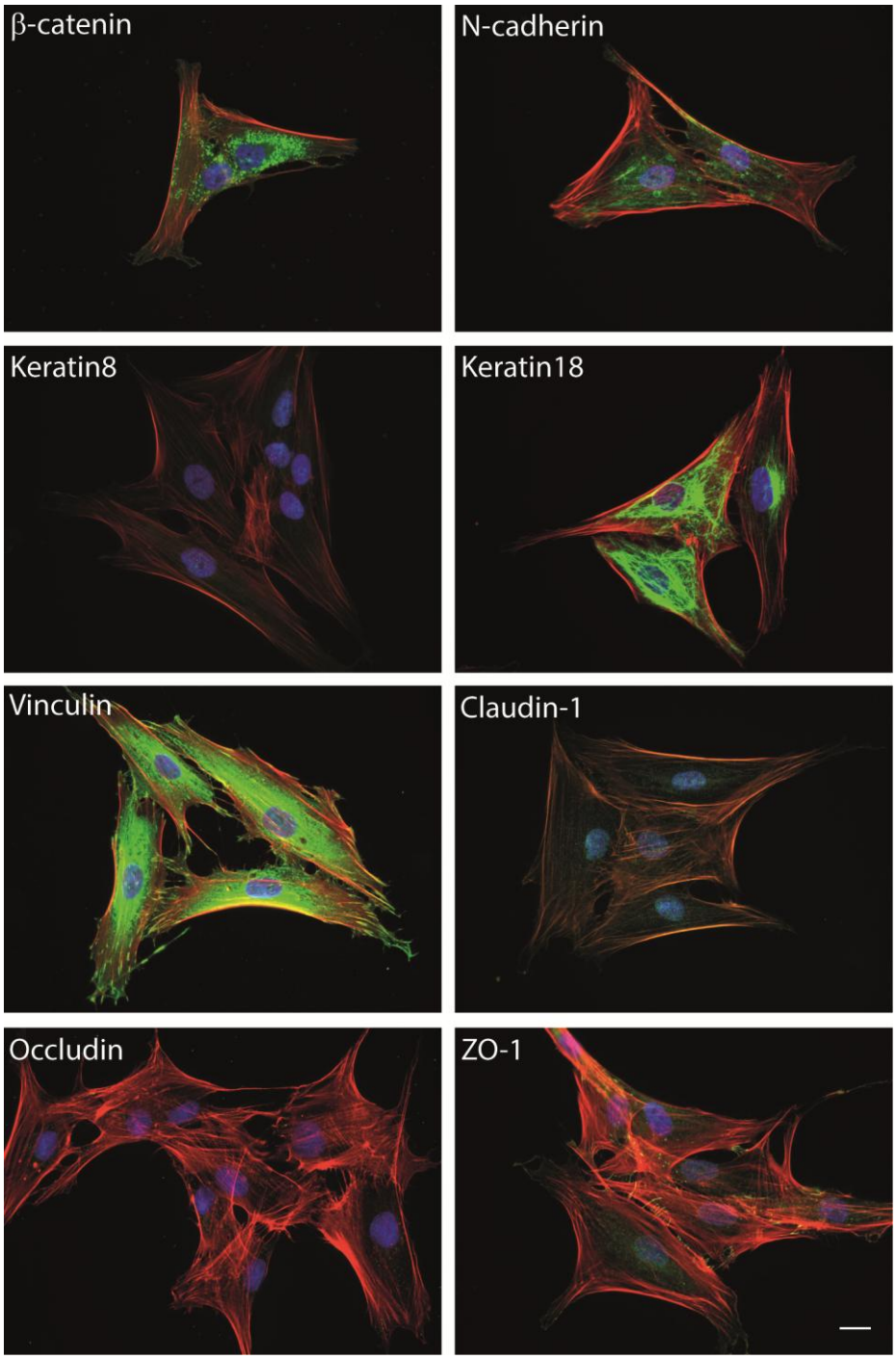


Figure 3.4: Protein expression in the duct epithelial cell line, dechTERT. To further characterise the dechTERT cell line, cells were stained for a variety of proteins including β-catenin, N-cadherin, CK8, CK18, vinculin, claudin-1, occluding and ZO-1 (green). Cells were co-stained with DAPI (blue) and actin (red). High levels of perinuclear β-catenin were observed as well as high global expression of CK18, vinculin and N-cadherin. Very little CK8, claudin-1 and occludin were observed. ZO-1 was observed in strands forming between cells. Scale bar = 10μm.

with epithelial to mesenchymal transition (EMT), which can be considered a hallmark of cancer invasion (Onder et al., 2008; Pecina-Slaus, 2003). In conjunction with a loss of E-cadherin, there is often a marked increase in the expression of N-cadherin observed; this event is known as cadherin-switching (Hazan et al., 2004; Maeda et al., 2005; Wheelock et al., 2008). As no E-cadherin was detected, the expression of N-cadherin was investigated. Cytoplasmic N-cadherin was observed in the dechTERT cells which implies a less epithelial-like cell.

It is known that pancreatic duct epithelial cells express cytokeratins (CK) 7, 8, 18 and 19 (Real et al., 1993; Santini et al., 1994) and so the dechTERT cells were also stained for the presence of CK8 and CK18. It was observed that they had only very low levels of CK8, but it appeared they had relatively high expression of CK18. The role of vinculin in cell-matrix adhesions is well documented (Zamir and Geiger, 2001; Zamir et al., 2000), but it is also known to impact on cell-cell adhesion (Bays et al., 2014; Peng et al., 2010). It was found that the dechTERT cells had global expression of vinculin, but also localised to adhesion like structures, as observed through the immunofluorescence studies. Some other intercellular adhesion proteins, which are important components of tight junctions, are claudins, occludins and zonula occludens (ZO) proteins (Schneeberger and Lynch, 2004; Tsukita et al., 2001). It has also been shown at either the protein or mRNA level that HPDE cells express several of these tight junction molecules including claudin-1, occludin and ZO-1 (Kojima and Sawada, 2012), so dechTERT cells were stained for these to further investigate their expression profile. It

appeared that there was no occludin present and only very low observed levels of expression of claudin-1. However, ZO-1 could be seen localised at finger-like projections, which appeared to form between cells.

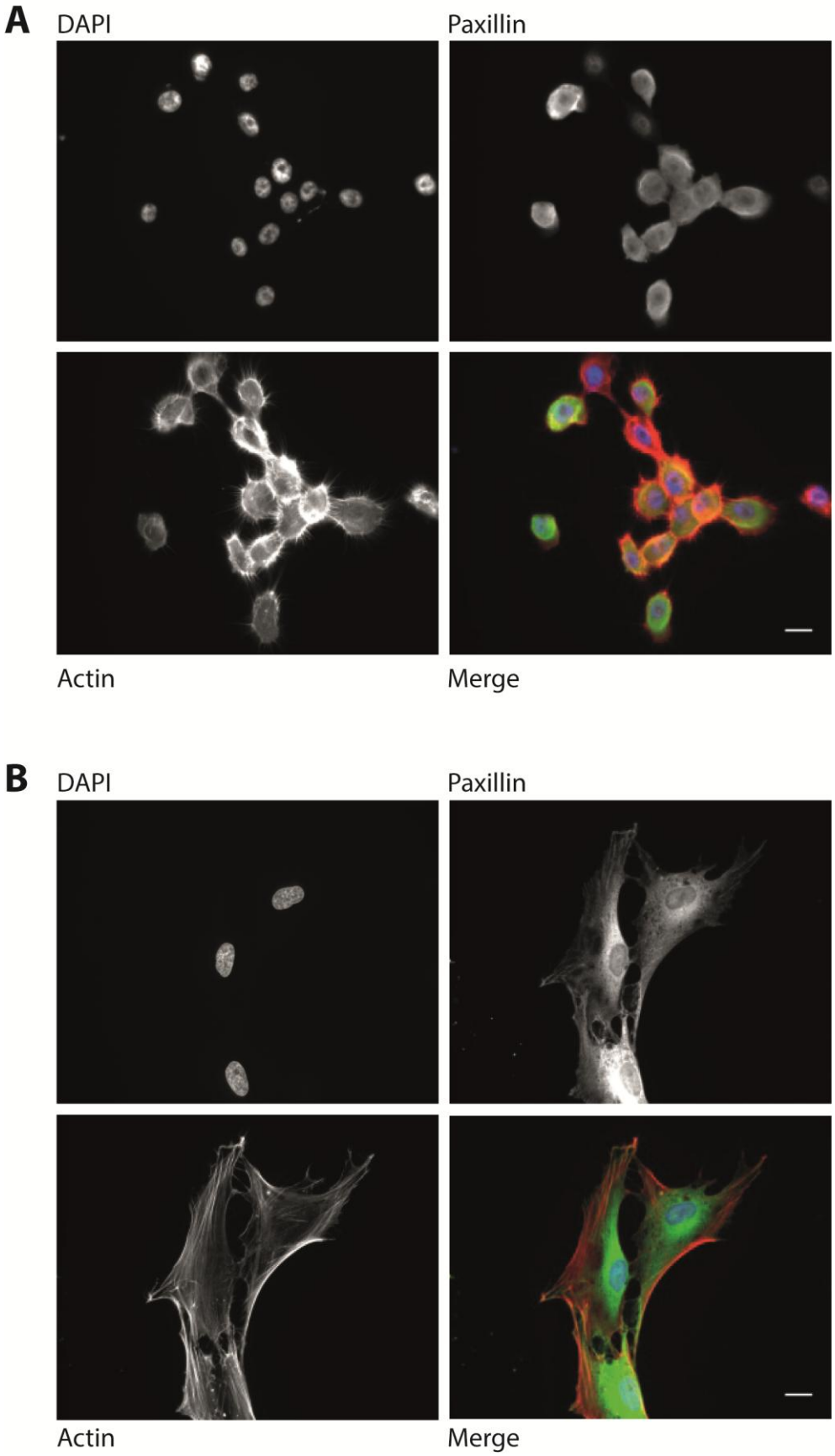
3.2.3 Morphology of pancreatic cell lines

To further characterise the cell morphology of each of the selected cell lines, where cells were seeded onto coverslips and subsequently stained for paxillin, actin and DAPI (**figure 3.5A, B, C** and **D**). HPDE cells were maintained in calcium, as before. Each cell lines was seeded onto uncoated, fibronectin and collagen (cancer cells only) coated coverslips in order to determine if this lead to any substantial morphological changes. Cells shape was analysed using ImageJ and the area of the cells was measured from three independent experiments. Analysis revealed that there was no significant difference between the measured parameters (**figure 3.5F**).

3.2.4 Characterisation of protein expression in pancreatic cell lines

In addition to immunofluorescent staining, the panel of pancreatic cell lines were analysed for differential protein expression. Whole cell lysates from HPDE cells were made from cells that were seeded both with and without calcium nitrate in order to determine if this impacted on protein expression.

Under normal cellular conditions, p53 performs crucial tumour suppressor functions, through the induction of cell cycle arrest of apoptosis in response to various cellular stresses (Brady and Attardi, 2010; Vousden and Prives, 2009). However, during oncogenic transformation, mutations occur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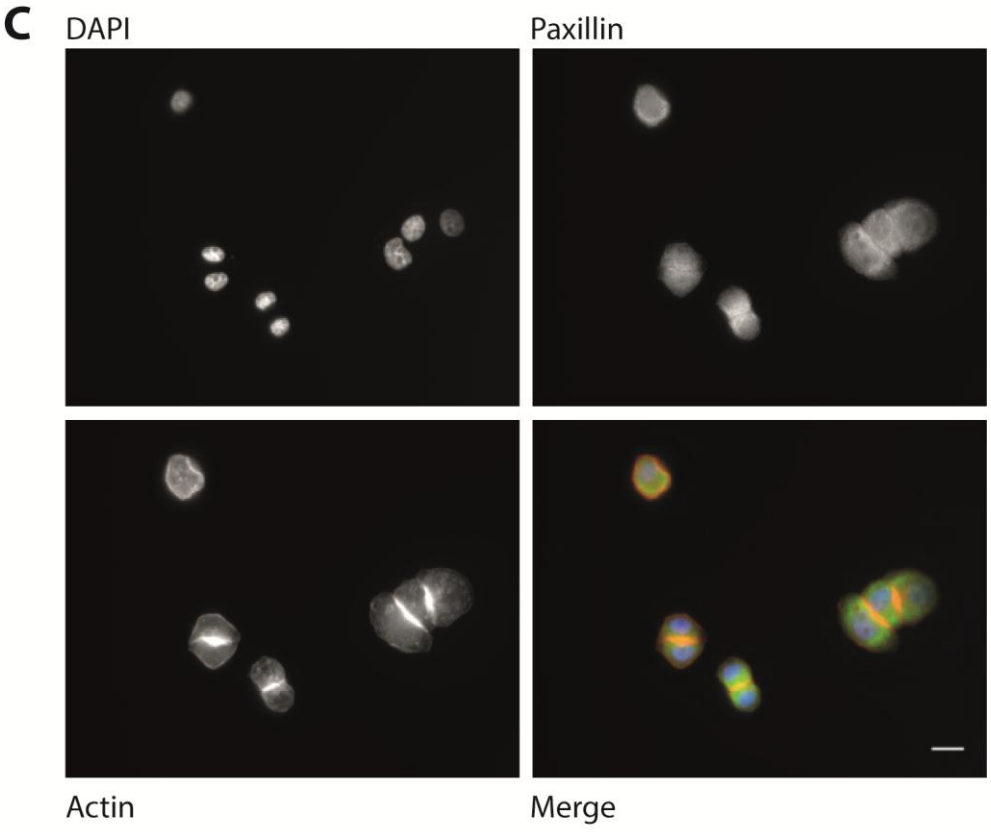
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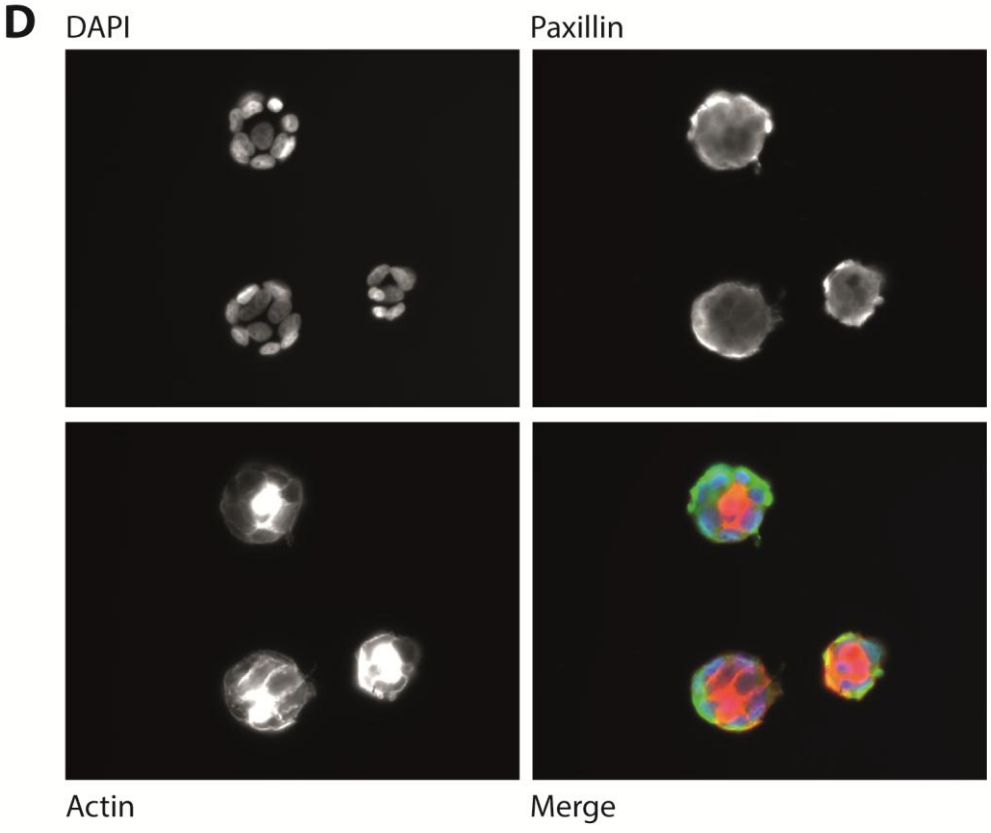
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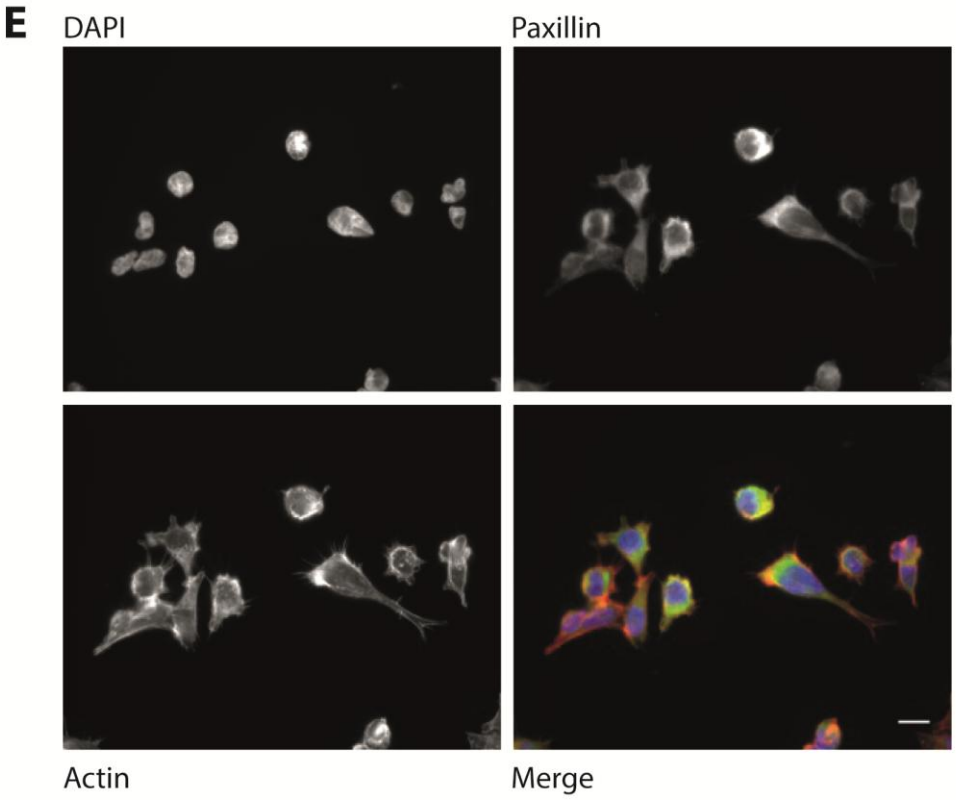
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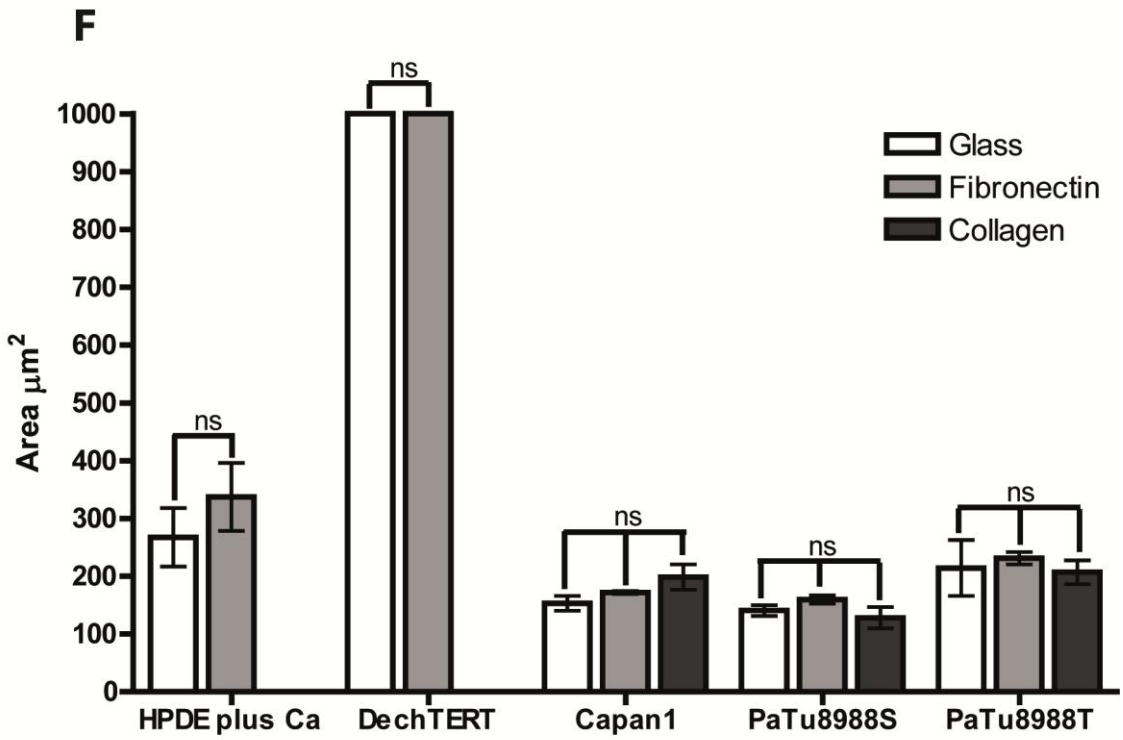
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Collagen
HPDE plus Ca
DechTERT
Capan1
PaTu8988S
PaTu8988T

Figure 3.5:Morphology of pancreatic cell lines. All cell lines being used were subjected to immunofluorescent staining in order to characterise cellular morphology on different substrates. HPDE (A), dechTERT (B), Capan1 (C), PaTu8988S (D) and PaTu8988T (E) cells were seeded onto glass (shown), collagen and fibronectin (both data not shown). They were stained with DAPI (blue), paxillin (green) and actin (red) and the area calculated using ImageJ, which was subsequently quantified (F). Results demonstrated that there was no significant different in overall cell area between any of the substrates used. Images and data were gathered from three independent experiments. Scale bar = 10μm.

commonly within the p53 gene, many of which result in the expression of a stable mutant protein. This subsequent accumulation within the cell is considered a hallmark of cancer (Muller and Vousden, 2013; Rivlin et al., 2011). It has also shown that there is a strong association between p53 expression and a transformed status (Moro et al., 1995) and so the status of p53 expression in the panel of pancreatic cell lines was investigated through western blotting (**figure 3.6A**). It was observed that there was a marked increased in p53 expression in the cancer cell lines in comparison to the epithelial cell lines, in line with the existing literature. Blots were subsequently quantified using ImageJ (**figure 3.6B**).

c-Met is known to have increased expression in pancreatic cancer (Herreros-Villanueva et al., 2012; Kiehne et al., 1997; Li et al., 2011a) and can lead to upregulation of the PI3K pathway. PI3K is made up of two main subunits, P110 and p85. It is known that the P110 subunit is overexpressed in human pancreatic cancer tissues and cell lines (Edling et al., 2010). However, within this study, the p85 subunit is of particular interest and therefore the expression of this protein and c-Met was investigated (**figure 3.7A**). The levels of both c-Met and p85 were increased in the cancer cell lines, in comparison to both HPDE and dechTERT epithelial cells. These blots were quantified through densitometry using ImageJ (**figure 3.7B**).

Like c-Met, K-ras is known to be a key component in the development and progression of pancreatic cancer (Collins et al., 2012). There are four Ras

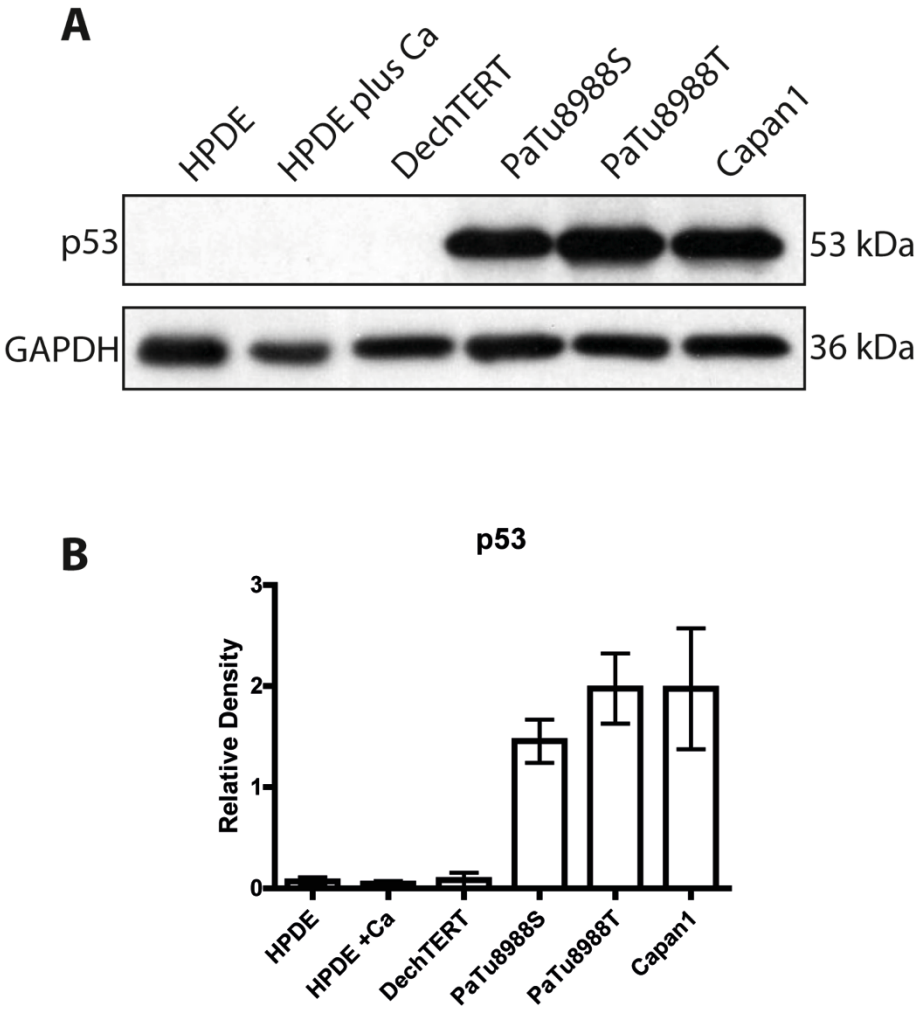


Figure 3.6: Expression of p53 in pancreatic cell lines. Western blotting was used to investigate the status of p53 expression in the panel of pancreatic cell lines (A). Both HPDE and dechTERT cells expressed virtually no p53 while the expression was greatly increased in the cancer cell lines. The blot shown is representative of three independent experiments, which were then subject to semi-quantitative analysis and densitometry (B), whereby each blot was quantified after normalising to the loading control (GAPDH). All three experimental repeats are represented in the graph depicted in (B).

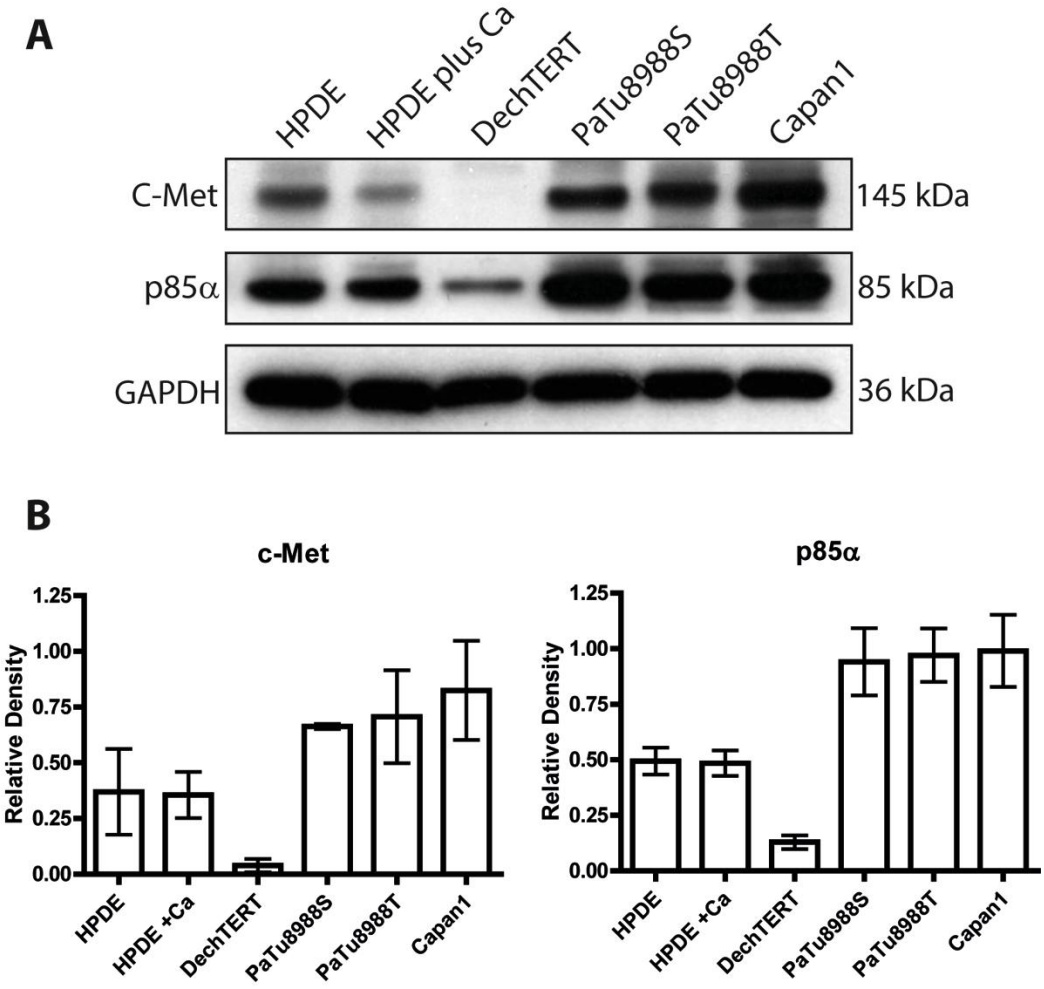


Figure 3.7: Expression of c-Met and p85α in pancreatic cell lines. Knowing the expression of c-Met in increased in pancreatic cancer, the relative expression of both c-Met and p85α (a subunit of PI3K, which lies downstream of c-Met) was investigated in order to determine the differential expression between normal pancreatic epithelia and pancreatic cancer cell lines. Western blotting (A) demonstrated an increase in the expression of both of these proteins in the cancer cells in comparison to both duct epithelial cell lines, which was confirmed through densitometry after normalising relative expression to the loading control (GAPDH) (B). Blots are representative of three independent experiments and the densitometry from each experimental repeat is depicted graphically (B).

isoforms (H-ras, N-ras, K-ras4A and K-ras4B), which have a highly conserved structure (**figure 3.8A**), with a C-terminal hypervariable region changing between the isoforms (Castellano and Santos, 2011). In order to ensure that the cell lysates were specifically blotted for K-ras expression, isoform specific antibodies were purchased and tested to check for specificity. Whole cell lysates from HEK293 cells, transfected with either myc tagged K-, H-, or N-ras, were generated and run in duplicate. Blots were then either probed with an anti-myc antibody, or the reported K-ras specific isoform antibody. The blots revealed that the antibody specifically recognized K-ras and there was no evidence cross-reaction (**figure 3.8B**). This antibody was subsequently used to detect the level of K-ras expression in the panel of pancreatic cell lines. Western blotting revealed a large increase in K-ras expression with increasing invasive potential (**figure 3.8C**), with the highest level being observed in the highly invasive PaTu8988T cell line. This pattern of expression was confirmed by densitometry (**figure 3.8D**) using ImageJ.

3.2.5 Characterisation of PAK family kinase expression

Having determined the expression profile of several proteins in the panel of pancreatic cell lines, the expression of both group I (1-3), and group II (4-6) PAKs was investigated. Although within this study, the main focus is on PAK4, all PAK expression was initially looked at.

The group I PAK expression was first investigated (**figure 3.9A**). There was a marked increase in all three group I PAKs in Capan1, PaTu8988S and PaTu8988T cells in comparison to both the HPDE and dechTERT cells, with

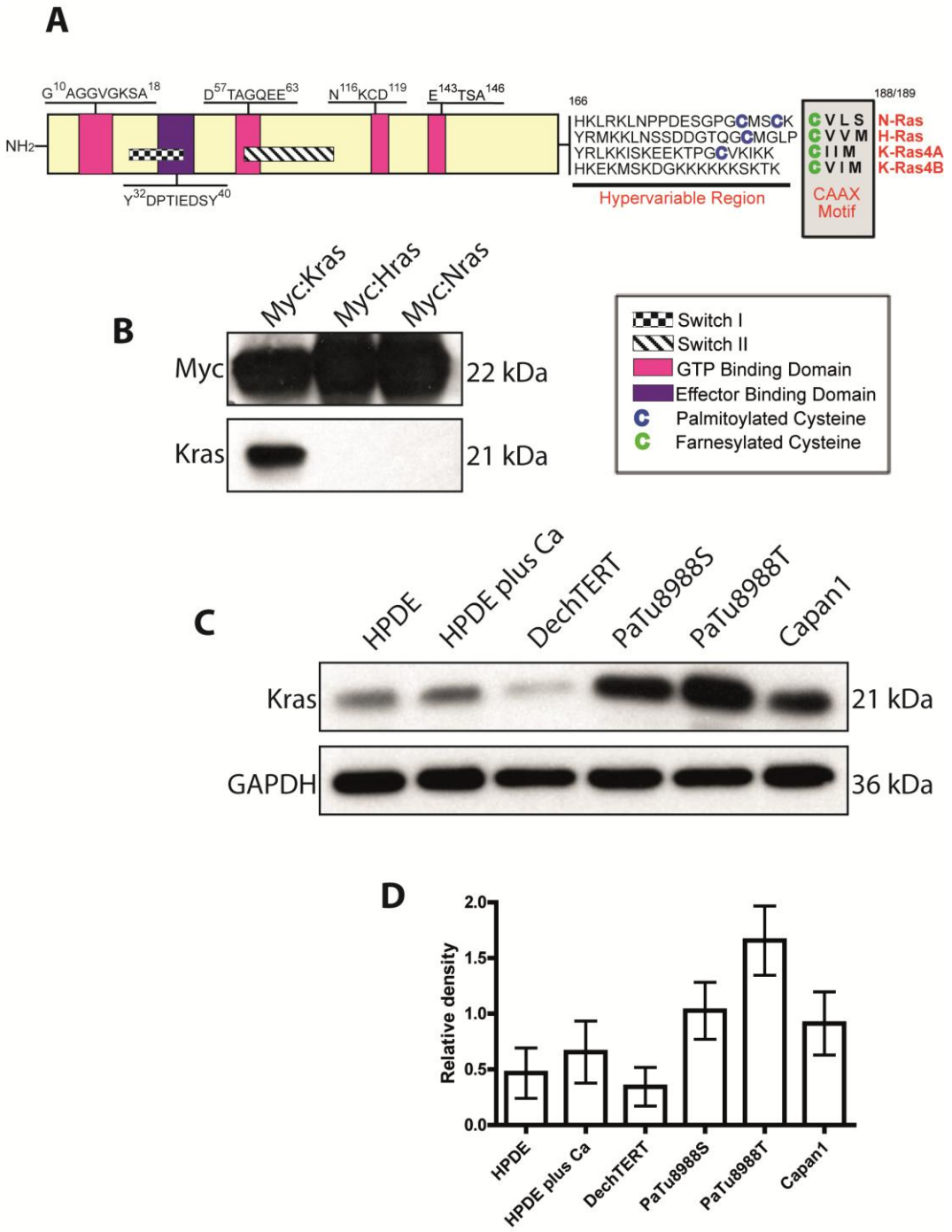


Figure 3.8: K-ras expression in pancreatic cell lines. Within the structure of Ras proteins (A), there are several highly conserved regions, including those for binding GTP/GDP and downstream effector proteins. There is a hypervariable region present on all four Ras isoforms, which contains a CAAX motif that is responsible for directing post-translational modifications such as farnesylation and palmitolation. (Figure adapted from: Castellano & Santos, 2011). Isoform specific antibodies were validated (B) by transfecting in myc-tagged Ras isoforms (K-, H- and N-ras) and probed for Myc and K-ras separately. Western blotting confirmed antibody specificity. This antibody was then used to probe the panel of pancreatic cell lines (C) and showed increased expression in the cancer cell lines in comparison to the epithelial cell lines, which was confirmed by densitometry (D) after normalising relative expression to the loading control (GAPDH).

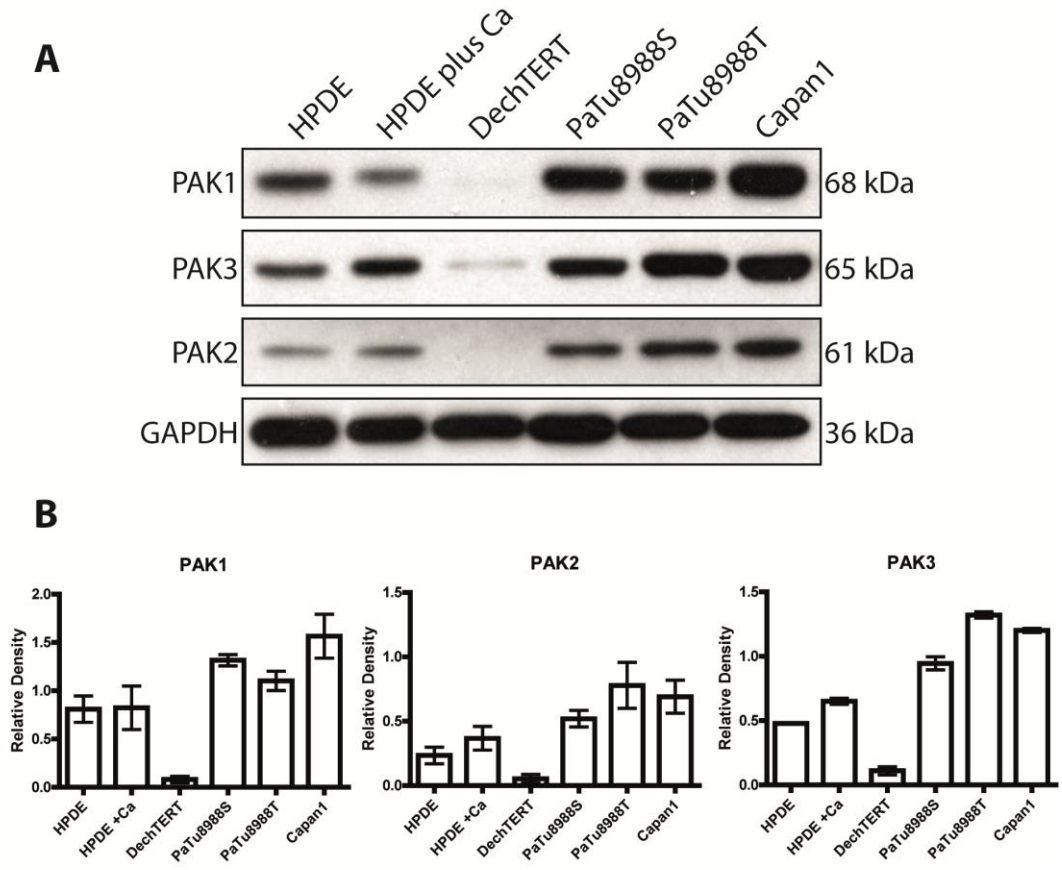


Figure 3.9: Expression of Group I PAKs in pancreatic cell lines. Western blotting was used to investigate the expression pattern on group I PAKs in the pancreatic cell lines (A). Each isoform had increased expression in the cancer cell lines, in comparison to the epithelial cell lines. The lowest expression was observed with PAK2. The blot shown is representative of three independent experiments, which were quantified through densitometry (B). Each experimental repeat was individually quantified and represented in graphical form after normalising to the loading control (GAPDH).

PAK2 appearing to having a relatively low level of expression. Group I PAK expression was also quantified through densitometry using ImageJ (**figure 3.9B**). The pattern of expression of group II PAKs was markedly different (**figure 3.10A**). While both PAK4 and PAK6 had increased expression in the cancer cell lines, PAK5 was expressed in the HPDE cells and both the colony forming cancer cell lines. From work in our lab, it has been suggested that PAK5 is present in colony forming cell lines and then expression is lost as cells become more invasive (unpublished data). The work carried out here compliments these previous studies as PAK5 was present in HPDE, Capan1 and PaTu8988S cells but PaTu8988T cells had no detectable PAK5 expression. PAK6 has been linked to prostate cancer invasiveness (Wen et al., 2009) and an increase in PAK6 expression leads to cell-cell dissociation in response to HGF (Fram et al., 2014). Results showed that there was increased expression of PAK6 with invasive potential, which compliments these previous studies. With regards to PAK4, it is known that it is oncogenic when overexpressed (Ahn et al., 2011; Liu et al., 2008a; Qu et al., 2001; Wong et al., 2013; Zhang et al., 2011a) and plays a significant role in oncogenesis. Therefore, it is not surprising to observe that there is a large increase in PAK4 expression in the cancer cell lines in comparison to the HPDE and dechTERT cells. The expression of group II PAKs was quantified through densitometry using ImageJ (**figure 3.10B**).

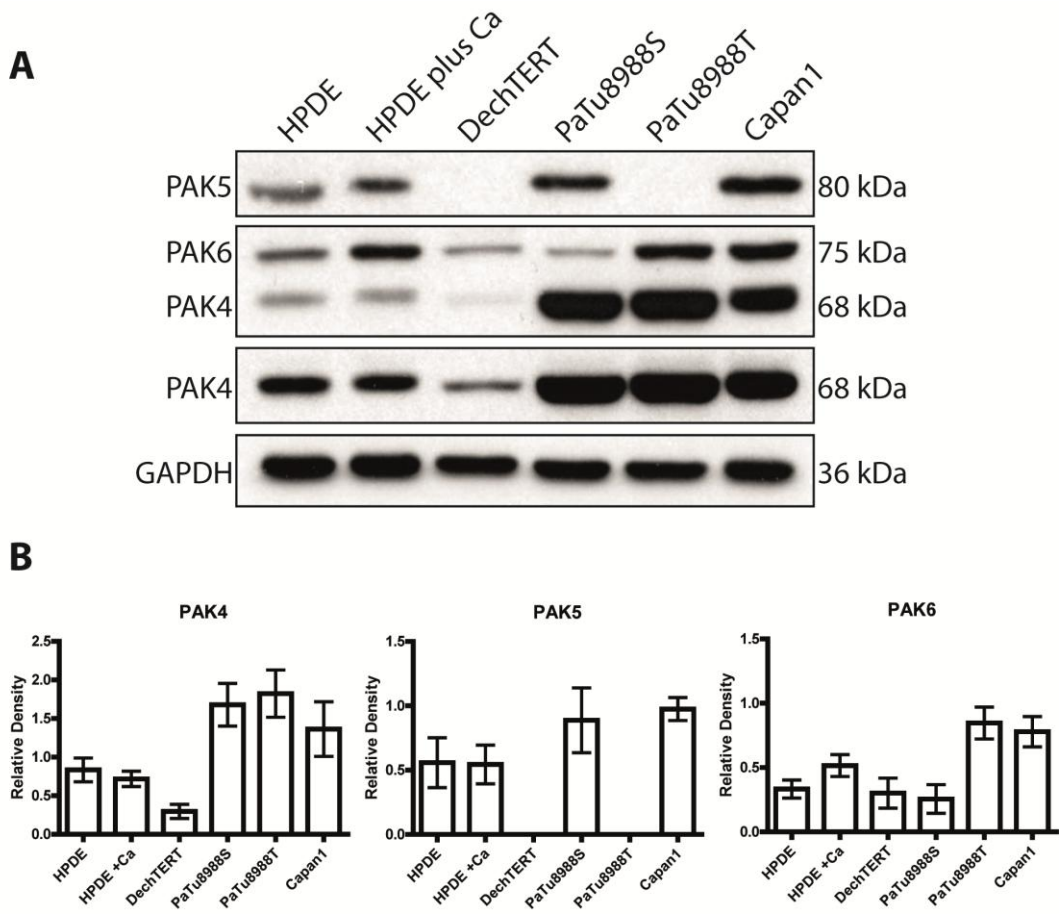


Figure 3.10: Expression of Group II PAKs in pancreatic cell lines. Western blotting was used to investigate the expression of group II PAKs in the pancreatic cell lines (A). It was noted that PAK5 was expressed in both colony forming cancer cell lines (PaTu8988S and Capan1) and in the HPDE cell line. PAK6 appeared to have higher expression in the invasive PaTu8988T cells in comparison to the PaTu8988S cell lines. PAK4 appeared to have the highest level of expression in all the cancer cell lines, in comparison to both epithelial cell lines. Blots shown are representative of three independent experiments, which were quantified through densitometry (B). Each experimental repeat was individually quantified and represented in graphical form after normalising to the loading control (GAPDH).

3.3 Discussion

In this chapter, a panel of pancreatic cell lines were characterised for cell morphology and protein expression patterns in order to determine the most appropriate cell lines to use going forward within the investigation.

Of the five cell lines used, there were two epithelial cell lines included within these initial studies, HPDE and dechTERT cells. It became apparent that these epithelial cell lines did not display typical epithelial cell characteristics including the formation of cell-cell junctions and/or the expression of epithelial markers. As such, a more thorough investigation was carried out to provide a more complete data set and understanding of these cells. Upon staining the HPDE cells with E-cadherin, it was shown that no junctional formation between cells was present. It was demonstrated though, that the addition of calcium nitrate induced the formation of cell contacts, with cells beginning to form monolayers. It was also shown that the addition of calcium nitrate to cells in culture had little impact on protein expression. The dechTERT cells, however, were much more complex. They are a duct epithelial derived cell line, which was initially characterised when they were first immortalised (Li et al., 2009). The initial studies here showed that they had a fairly complex expression profile. These cells expressed no E-cadherin and immunofluorescence studies demonstrated that N-cadherin was present. This pattern of cadherin expression is characteristic of cells that have undergone cadherin switching during EMT (Hazan et al., 2004; Maeda et al., 2005). Also in line with this data, was the high level of β-catenin expression, which was concentrated in the perinuclear region, as is expected of cells not

expressing E-cadherin, which retains β-catenin within the region of the plasma membrane (Orsulic et al., 1999). It is within this cellular region that it aids in the formation of intercellular junctional stability, forming part of the link between E-cadherin and the actin cytoskeleton (Desai et al., 2013; Valbuena et al., 2012). Once in the cytoplasm, it is known that β-catenin can translocate into the nucleus and subsequently partake in Wnt signaling, which is involved in cancer progression and cytoplasmic accumulation is frequently observed in squamous cell carcinoma (Iwai et al., 2010). It is also known that disregulation of the β-catenin pathway can be correlated with EMT and invasiveness (Prasad et al., 2008; Schmalhofer et al., 2009). These E-cadherin and β-catenin studies therefore indicated that the dechTERT cells may not be completely representative of normal panctreatic duct epithelia.

Within the characterisation of the dechTERT cells, they were also shown to have expression of some junctional proteins, such as ZO-1 but lacked expression of others including claudin-1, both of which were found in another hTERT immortalised duct epithelial cell line in previous studies (Kojima and Sawada, 2012). They also had high expression of CK18 but not CK8, however, both of these cytokeratins can be found in both cancer and duct epithelial cells (Real et al., 1993; Santini et al., 1994) and thus may not be the optimal epithelial markers to have used within this study. Taken altogether, these results suggest a protein expression profile that was not necessarily indicative of epithelial cells. However, it has been shown that pancreatic ductal cells can dedifferentiate and act as pancreatic progenitors and this dedifferentiation can be associated with EMT, with cells co-

expressing epithelial (cytokeratin7 and cytokeratin19) and mesenchymal (vimentin and α-smooth muscle actin) markers (Fanjul et al., 2010). Therefore, it could be of interest to investigate the expression of these markers in the dechTERT cells, also to include markers of progenitor pancreatic cells, such as pancreatic duodenal homeobox factor-1 (Fanjul et al., 2010).

Another objective of these preliminary studies was to determine if substrate variation lead to any pronounced changes in the cellular morphology. The extracellular matrix is a complex structure and formulated from a dynamic range of proteins and growth factors (Kim et al., 2011b; Taipale and Keski-Oja, 1997). This can lead to changes in cellular behavior and/or morphology, which is dependent on receptor expression that can vary between cell lines (Kiss et al., 2013; Legate et al., 2009). It was therefore decided to test a range of matrix substrates and look at cellular morphology on each. Cell area was measured after seeing on uncoated and fibronectin or collagen coated coverslips. It was found that there was no significant difference in morphology or area. From this, it was subsequently decided to perform future immunofluorescence/migration assays on a collagen I matrix, as collagen I is a prominent component in the extracellular matrix and heavily involved in the desmoplastic/fibrotic reaction associated with PDAC (Shields et al., 2011) and was also one of the main constituents of the organotypic model which is planned for later functional studies.

Having established the epithelial relevance of the HPDE +/- calcium and dechTERT cells, these cells were subsequently tested against several pancreatic cancer cell lines for differential expression of project interest proteins. This was to observe how the expression varied between epithelial, non-invasive and invasive cancer cell lines. Firstly, HPDE cells were studied both with and without the addition of calcium nitrate in order to determine if the addition lead to any changes in protein expression. However, there were no significant changes in any of the proteins investigated throughout the course of these initial studies. Of all the proteins under examination there was increased expression in the cancer cells in comparison to the epithelial cells lines. Many of these proteins are known to be important players in pancreatic cancer, including c-Met and K-ras and so it is expected that these would have a higher level of expression in these cancer cell lines.

With regards to PAK expression, there has been much research into how they contribute to disease progression and cancer (King et al., 2014). However, there is little reported research on the impact of PAK expression specifically in pancreatic cancer. Although more recently one study, published during this project, did implicate PAK1 as having a role in pancreatic tumorigenesis (Jagadeeshan et al., 2014) and tentative links between the role of PAK4 in PDAC progression have been made (Chen et al., 2008; Kimmelman et al., 2008).

With regards to group I PAKs, it was noted that not only PAK1, but also PAK3 appeared to have increased expression. This is perhaps unexpected

as PAK3 is primarily expressed in neuronal tissues, playing a crucial role in the development of normal brain functioning (Huang et al., 2011) and loss of function mutations being linked to mental retardation and/or learning difficulties (Dubos et al., 2012; Kreis et al., 2007). However, it has been shown that PAK3 is present in thyroid cancer tissue samples and cell lines (McCarty et al., 2010). PAK2 appeared to have low expression across the panel of cell lines tested, however, there was still an increase in the expression of PAK2 in the three cancer cell lines in comparison to the HPDE cells. A recent study demonstrated that ovarian cancer cells had increased migrational capacity on a collagen I matrix, which was mediated (at least in part) by PAK2 (Flate and Stalvey, 2014). As collagen I is known to be one of the main constituents of the ECM, which plays a significant role in pancreatic cancer (Shields et al., 2011), it would be of interest to investigate whether PAK2 has a similar role in pancreatic cancer.

Of the group II PAKs, PAK4 had a notable increase in expression between the epithelial and cancer cell lines. It is known that PAK4 plays a major role in oncogenesis, being implicated in a number of cancers including breast (Callow et al., 2002; Liu et al., 2010b; Liu et al., 2008a; Minden, 2012), prostate (Ahmed et al., 2008; Park et al., 2013; Whale et al., 2013), colon (Tabusa et al., 2013), ovarian (Davis et al., 2013; Siu et al., 2010a), as well as pancreatic (Chen et al., 2008; Kimmelman et al., 2008; Mahlamaki et al., 2004). It was also demonstrated that PAK5 had expression within the colony forming cell lines, but not in the highly invasive PaTu8988T cells. Previous work in our lab (currently unpublished) suggests PAK5 displays increased

expression in colony forming cells of the bladder and breast as well as the pancreas. This leads to the prediction that loss of PAK5 could therefore lead to increased invasion of cancer cells, the opposite effect of both PAK4 and PAK6. Indeed, western blotting within this study showed that there was an increase in PAK6 expression in the PaTu8988T cell line, in comparison to the matched, colony forming cells PaTu8988S. This compliments previous work from our lab using the prostate cancer cell line DU145, which demonstrated a link between PAK6 expression and cancer cell invasiveness, with overexpression of PAK6 leading to colony dissemination (Fram et al., 2014).

It was found during these preliminary studies that both PaTu8988T and PaTu8988S had the highest level of PAK4 expression across all cell lines tested. This lead to the decision to continue using this matched pair throughout the course of this investigation. This would allow comparison between a non-invasive and invasive phenotype within pancreatic cancer. It was decided against using either epithelial cell line as an additional control due to the lack of typical epithelial behaviour and protein expression.

In summary, it was confirmed that there was increased expression of key components of pancreatic tumorigenesis and high PAK4 expression levels in pancreatic cancer. These data support further investigation of the role PAK4 may play within the development and progression of this disease.

3.4 Future Work

Studies of disease progression are greatly enhanced by the presence of a normal/control cell line. Therefore, in the future, if the dechTERT cells were to be considered for this use it would be beneficial to further characterise this cell line. Due to time constraints, the protein expression profile performed as part of the preliminary work is still incomplete. It would be advisable to perform further western blotting and immunofluorescence (or an array to allow for a larger screen) for additional epithelial/mesenchymal markers to build a more complete picture as to the exact nature of these cells. As they also do not exhibit the typical epithelial morphology, it may be advised to perform some putative random migration assays.

In this chapter, it was also shown that the expression of PAKs 1, 2, 3 and 6, as well as 4 were increased in cancer cell lines, in comparison to HPDE and dechTERT cells. It could therefore be considered interesting, particularly regarding PAK6 where there was increased expression in the invasive cell line, in comparison to the non invasive sister cell line or PAK2 which has been shown to influence collagen I mediated cell migration, to follow up as to whether they play a role in pancreatic cancer in addition to the work done on PAK4 within this study.

Chapter 4
Results: Part 2
Characterisation of PAK4 depletion in pancreatic cancer cell lines

Chapter 4: Characterisation of PAK4 depletion in pancreatic cancer cell lines

4.1 Introduction

The c-Met proto-oncogene codes for the c-Met protein, which is a receptor tyrosine kinase. It is a cell surface receptor, and can be found expressed in a range of epithelial cells including those of the prostate, liver and pancreas both during development and throughout adulthood (Organ and Tsao, 2011). Post-translational, proteolytic processing produces a single-pass, disulphide-linked α/β heterodimer (Comoglio et al., 2008; Organ and Tsao, 2011). This is the receptor for HGF, which is also known as scatter factor (Naldini et al., 1991; Weidner et al., 1991). Upon binding to c-Met, HGF acts as a pleiotropic factor and cytokine leading to promotion of proliferation, cell survival, motility, differentiation, morphogenesis and scattering (Boros and Miller, 1995; Brinkmann et al., 1995; Zhu et al., 1994). HGF/c-Met signalling is known to play an important role in embryogenesis and wound repair through induction of cell scattering involving the dissolution of cadherin-based cell junctions and increased cellular motility (Chmielowiec et al., 2007).

Upon binding, HGF induces c-Met receptor dimerisation and a phosphorylation cascade, leading to the induction of downstream signalling (Blumenschein et al., 2012). There are two main arms of HGF/c-Met signalling, which are the MAPK and PI3K pathways. The PI3K pathway can be activated downstream of Ras activity or can be stimulated either directly or indirectly (via Gab1) through the p85 subunit of PI3K binding the c-Met receptor (Organ and Tsao, 2011). Activation of the c-Met signalling

pathways, leads to the phosphorylation of downstream signalling proteins including ERK and Akt (Tanahashi et al., 2013).

It has been demonstrated that PAK1 is overexpressed in pancreatic cancer tumour samples and that it plays a key role in PDAC signalling, acting downstream of HGF and c-Met to drive cancer cell motility (Zhou et al., 2014). It is also known that ras activates PAK1 via PI3K-dependent pathways (Dummler et al., 2009) and that a reduction in PAK1 expression leads to decreased ERK and Akt activity in colon cancer (Huynh et al., 2010). Also within colon cancer, it was shown that siRNA induced depletion of PAK4 lead to a reduction in Akt phosphorylation (Tabusa et al., 2013), although the authors stated that results were not consistent across a range of RNAi oligonucleotides tested. Another study provided further evidence supporting the hypothesis that PAK4 may lie within the PI3K pathway downstream of ras, which demonstrated that in NIH3T3 PAK4 knockout cells there was a reduction in Akt phosphorylation at Ser473. This phenotype was rescued through the introduction of K-ras V12 (Gnad et al., 2013).

We, and others, have found PAK4 to be overexpressed in pancreatic cancer cell lines (Kimmelman et al., 2008). It is also known to contribute heavily to oncogenesis, particularly in cancer cell motility and through modulation of the actin cytoskeleton, it promotes migration and invasiveness (Whale et al., 2013). It has been shown that constitutively active PAK4 leads to increased invasiveness of pancreatic ductal cells, with siRNA mediated depletion of PAK4 within PaTu8988T cells resulting in a reduction of anchorage

independent growth (Kimmelman et al., 2008). It has also previously been shown that PAK4 interacts with LIMK, enhancing its ability to phosphorylate cofilin, inhibiting its actin disassembly function (Dan et al., 2001). This interaction is known to be downstream of c-Met in prostate cancer in a HGF-dependent manner (Wells et al., 2002), with PAK4 depleted prostate cancer cells being less responsive to HGF (Wells et al., 2010; Whale et al., 2013).

Although typically the PI3K/Akt pathways has been considered primarily to be responsible for survival signalling, there is accumulating evidence to suggest that PI3K/Akt signalling contributes to cellular motility, including in metastatic cancer cells (Xue and Hemmings, 2013). Although there have been more recent publications linking PAK4 with PI3K and K-ras, the nature of this relationship within pancreatic cancer has yet to be fully elucidated. Therefore, knowing that PAK4 activity is at least in part reliant on c-Met and PI3K activity and that both are upregulated in pancreatic cancer, the nature of this relationship was further interrogated within this chapter. The chapter discusses the impact on HGF on PaTu8988S/T signalling and migration and looked to investigate further the effect of PAK4 knockdown on pancreatic cancer cell motility, as well as looking at downstream effectors of PAK4 signalling in pancreatic cancer cells.

4.2 Results

4.2.1 HGF leads to increased AKT and ERK phosphorylation

Results from the previous chapter reveal an increased expression of the c-Met receptor tyrosine kinase in both PaTu8988S and PaTu8988T cells. c-Met acts as a high affinity receptor for HGF, and signalling through this receptor is key to a number of biological processes including development, wound healing and morphogenesis (Maulik et al., 2002). HGF/c-Met signalling is known to play a significant role in pancreatic tumorigenesis too, acting upstream of several signalling pathways including PI3K and MAPK (Maehara et al., 2001; Patel et al., 2014; Tan and Yang, 2010). It is also known that there are increased levels of circulating HGF in pancreatic cancer patients (Kemik et al., 2009), so the effect of HGF stimulation on downstream effectors was investigated in the selected cell lines. Both PaTu8988S (**figure 4.1A**) and PaTu8988T (**figure 4.1C**) were serum starved, subjected to HGF stimulation for set time point and lysates made. Western blotting revealed that upon HGF stimulation, there was an increase in both the phosphorylation of Akt and ERK, which was evident after 5 minutes. In both cell lines, Akt peaked after 5 minutes, with no further increase in the phosphorylation being detected. ERK, however, peaked at 10 minutes. These results were confirmed through densitometry (**figure 4.1Bi**, **4.1Bii**, **4.1D** and **4.1Dii**).

4.2.2 HGF has no effect on the phosphorylation of group II PAKs

Until more recently, there was little knowledge on the regulation of PAK4. It was thought that phosphorylation at a specific serine residue (Ser474) was a

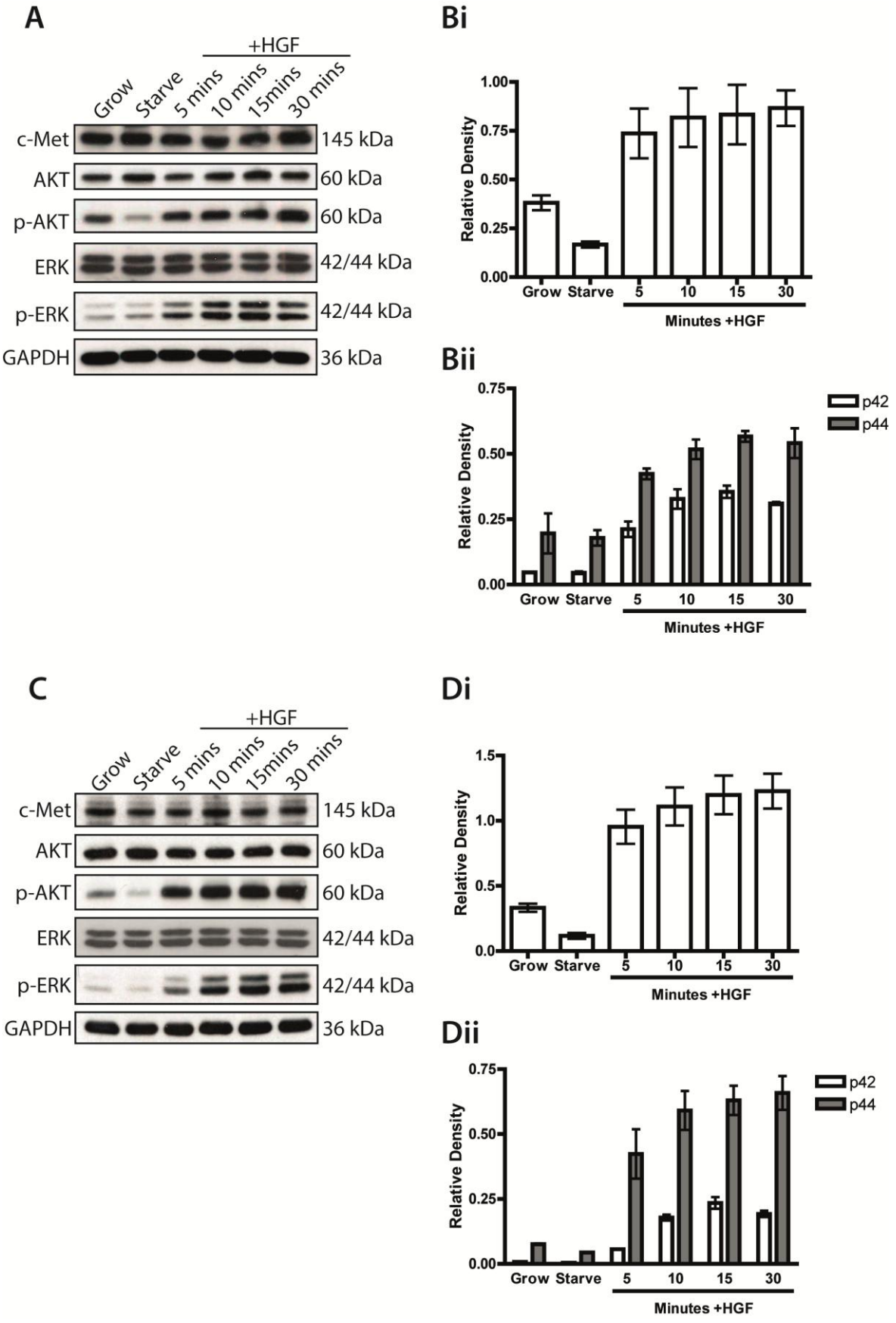

A
+HGF
Grow
Starve
5 mins
10 mins
15mins
30 mins
c-Met
145 kDa
AKT
60 kDa
p-AKT
60 kDa
ERK
42/44 kDa
p-ERK
42/44 kDa
GAPDH
36 kDa
Bi
Relative Density
1.00
0.75
0.50
0.25
0.00
Grow Starve 5 10 15 30
Minutes +HGF
Bii
Relative Density
0.75
0.50
0.25
0.00
p42
p44
Grow Starve 5 10 15 30
Minutes +HGF
C
+HGF
Grow
Starve
5 mins
10 mins
15mins
30 mins
c-Met
145 kDa
AKT
60 kDa
p-AKT
60 kDa
ERK
42/44 kDa
p-ERK
42/44 kDa
GAPDH
36 kDa
Di
Relative Density
1.5
1.0
0.5
0.0
Grow Starve 5 10 15 30
Minutes +HGF
Dii
Relative Density
0.75
0.50
0.25
0.00
p42
p44
Grow Starve 5 10 15 30
Minutes +HGF

Figure 4.1: Effect of HGF stimulation on the phosphorylation of AKT and ERK. Both PaTu8988S (A) and PaTu8988T (B) cells were subjected to stimulation with HGF where cells were starved overnight before HGF was added at specific time point. Lysates were separated by SDS-PAGE and western blotting revealed that there was an increased in the phosphorylation of both AKT and ERK, which was confirmed through densitometry (Bi, Bii, Di and Dii). Blots are representative of three independent experiments. Each experimental repeat was individually quantified using ImageJ and represented in graphical form after normalising to the loading control (GAPDH).

sign of its activation. However, more recent reports suggested that it was constitutively phosphorylated at this site and that its regulation was controlled by either an autoinhibitory domain, or a pseudosubstrate region within the N-terminal (Baskaran et al., 2012; Ha et al., 2012). Knowing that the PaTu8988T cell line responded to HGF and previous work in our lab suggested that PAK4 was activated downstream of HGF (Ahmed et al., 2008; Wells et al., 2002), it was decided to look at the phosphorylation of PAK4 after cells were stimulated with HGF. Western blotting analysis revealed that in line with the more recent literature, no change in PAK4 phosphorylation was observed after stimulating the cells with HGF (**figure 4.2**).

4.2.3 HGF leads to an increase in pancreatic cancer cell migration

Having confirmed that both the pancreatic cancer cell lines (PaTu8988S and PaTu8988T) responded to HGF through an increase in the phosphorylation of Akt and ERK, the migratory response was next investigated. HGF (also called scatter factor) is known to have mitogenic, motogenic and chemoattractant activities (Giacobini et al., 2007; Stella and Comoglio, 1999) and that both the circulating levels of HGF and its receptor, c-Met are increased in pancreatic cancer (Ebert et al., 1994). Random 2D migration assays were carried out with both PaTu8988S and PaTu8988T cells. PaTu8988S cells form very tight colonies, with high expression of E-cadherin concentrated at the junctions between cells. They are also a non-invasive cell line. It is known that HGF can lead to dissolution of cell contacts (Royal et al., 2000; Wells et al., 2002), however, it appear to have little impact on

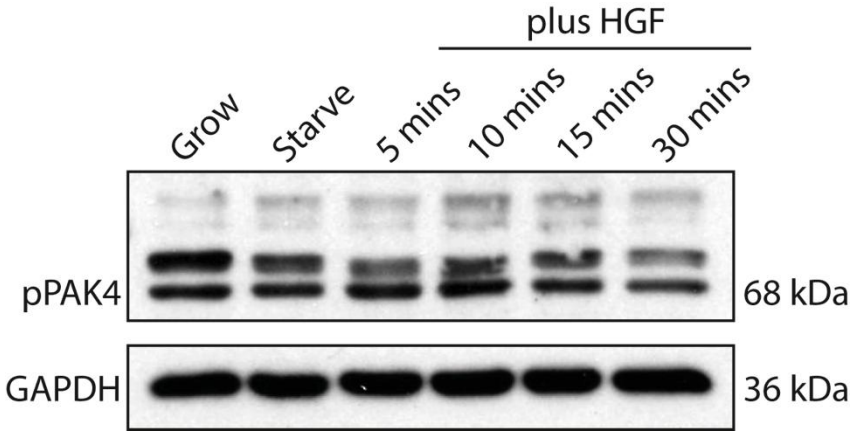


Figure 4.2: Effect of HGF on the phosphorylation of group II PAKs. PaTu8988T cells were subjected to HGF stimulation after being maintained in starve media (0% FBS) overnight. Lysates were produced after exposure to HGF for set time periods (between 5-30 minutes). A group II PAK phospho antibody was used and revealed that there was no change in the phosphorylation of PAK4 (indicated). Additional bands are phospho-PAK6 and phospho-PAK5 which also show that there is no change in phosphorylation status after stimulating the cells with HGF.

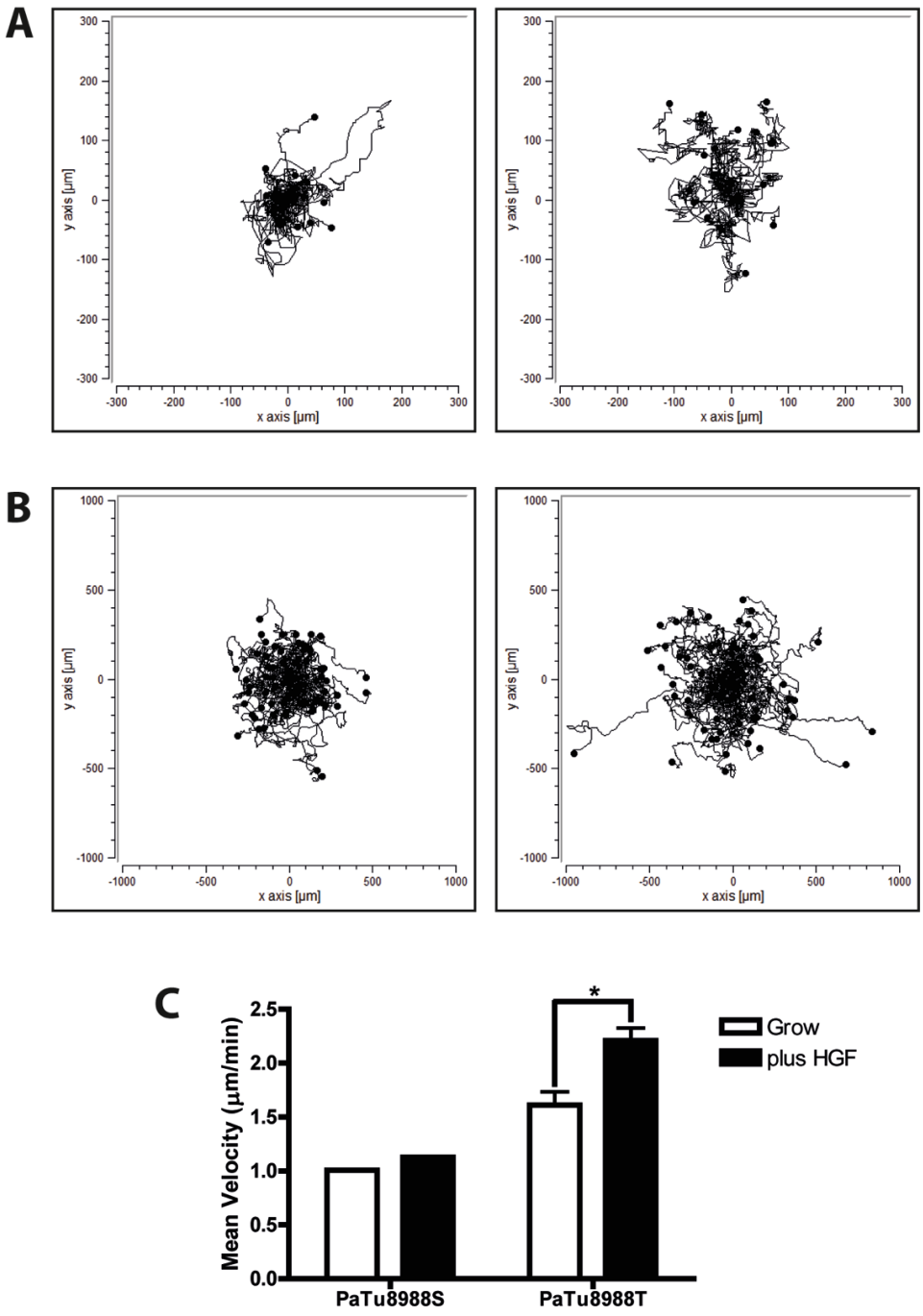
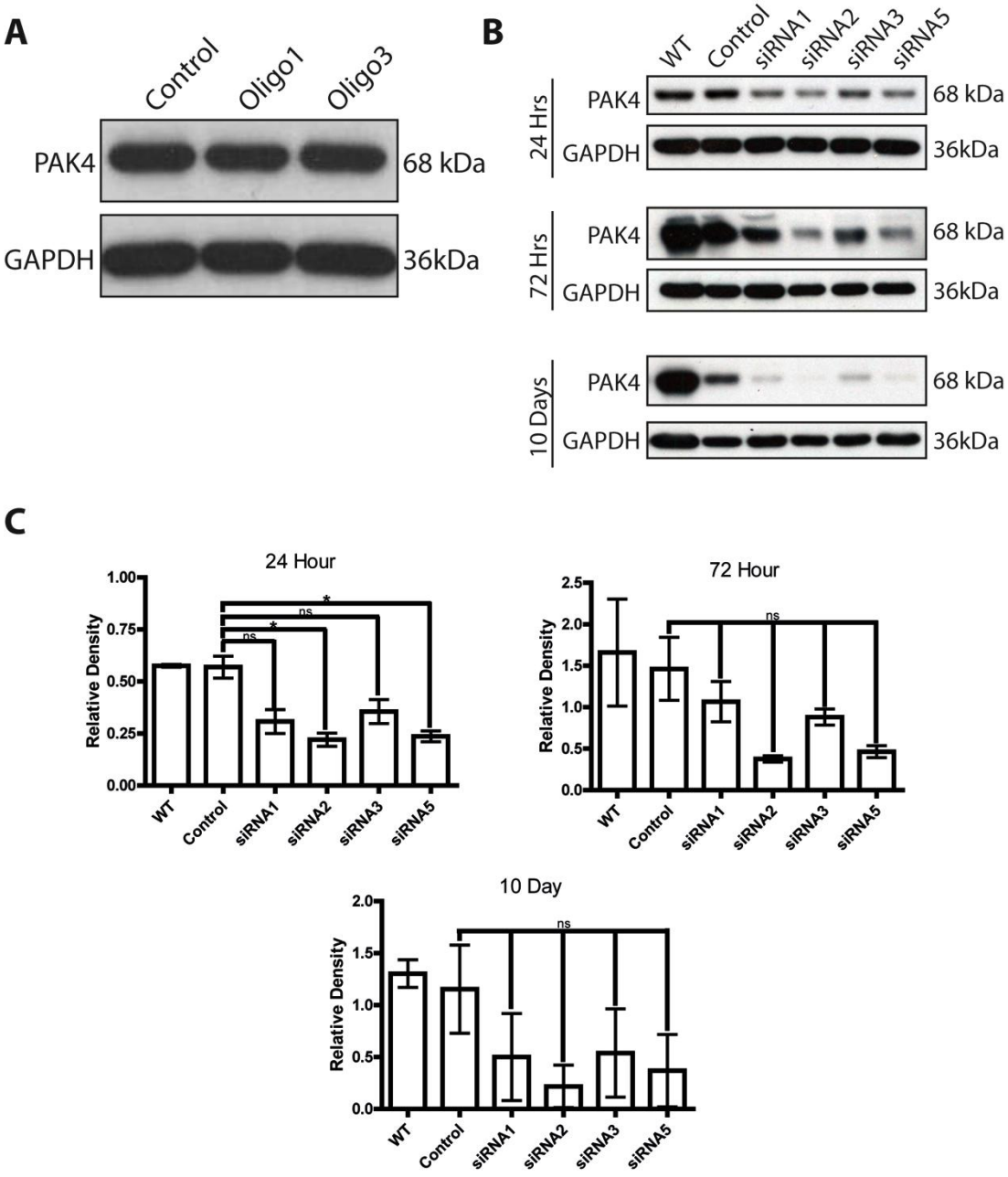


Figure 4.3: Effect of HGF on PaTu8988S/T cell migration. Both PaTu8988S and PaTu8988T cells were analysed for the change in migration rates after the addition of HGF (Experiments were performed in serum-containing media +/- HGF). PaTu8988S cells (A) had a very low rate of migration with little change observed after the addition of HGF. For PaTu8988S cells, N=1. PaTu8988T cells are an invasive cell lines and the mean velocity increased after HGF (B). Mean velocities were calculated (C) and PaTu8988T cells demonstrated a significant increase in velocity after HGF stimulation. Results from PaTu8988T cells are from three independent experiments. * $P<0.05$

PaTu8988S cell morphology or colony formation. Cells were tracked and plot profiles produced (**figure 4.3A**) and mean velocity calculated (**figure 4.3C**). PaTu8988S cells migrated very little, remaining in colonies and little change was observed after the addition of HGF. PaTu8988T cells are a highly invasive cell line (Marques et al., 2009), which was shown within this assay. Plot profiles were produced after tracking cells (**figure 4.3B**) and the mean velocity calculated (**figure 4.3C**). There was a significant increase of almost 40% in average cell velocity after cells had been subjected to HGF stimulation.

4.2.4 PAK4 knockdown in PaTu8988S and PaTu8988T cells

Knowing that PAK4 is overexpressed in these cell lines and has been implicated in pancreatic tumorigenesis (Chen et al., 2008; Kimmelman et al., 2008), the effect of depleting these cells of PAK4 expression was next investigated. It was originally planned to use an shRNA vector that has previously been used in the lab to produce a stable PAK4 knockdown cell line (Whale et al., 2013). However, even after adding the antibiotic selection and achieving a transfection efficiency of approximately 90%, PAK4 expression remained unchanged (**figure 4.4A**). It was therefore decided to move to using siRNA in order to knockdown PAK4 in both PaTu8988T and PaTu8988S cells. Four different siRNA oligonucleotides were purchased and trialed in order to determine which resulted in the greatest reduction of PAK4 protein expression and how long these effects would be maintained. A non-targeting control siRNA as well as untransfected/wild type (WT) cells were used as controls.


A
Control
Oligo1
Oligo3
PAK4
68 kDa
GAPDH
36kDa
B
WT
Control
siRNA1
siRNA2
siRNA3
siRNA5
24 Hrs
PAK4
68 kDa
GAPDH
36kDa
72 Hrs
PAK4
68 kDa
GAPDH
36kDa
10 Days
PAK4
68 kDa
GAPDH
36kDa
C
24 Hour
Relative Density
1.00
0.75
0.50
0.25
0.00
*
ns
*
ns
WT
Control
siRNA1
siRNA2
siRNA3
siRNA5
72 Hour
Relative Density
2.5
2.0
1.5
1.0
0.5
0.0
ns
WT
Control
siRNA1
siRNA2
siRNA3
siRNA5
10 Day
Relative Density
2.0
1.5
1.0
0.5
0.0
ns
WT
Control
siRNA1
siRNA2
siRNA3
siRNA5

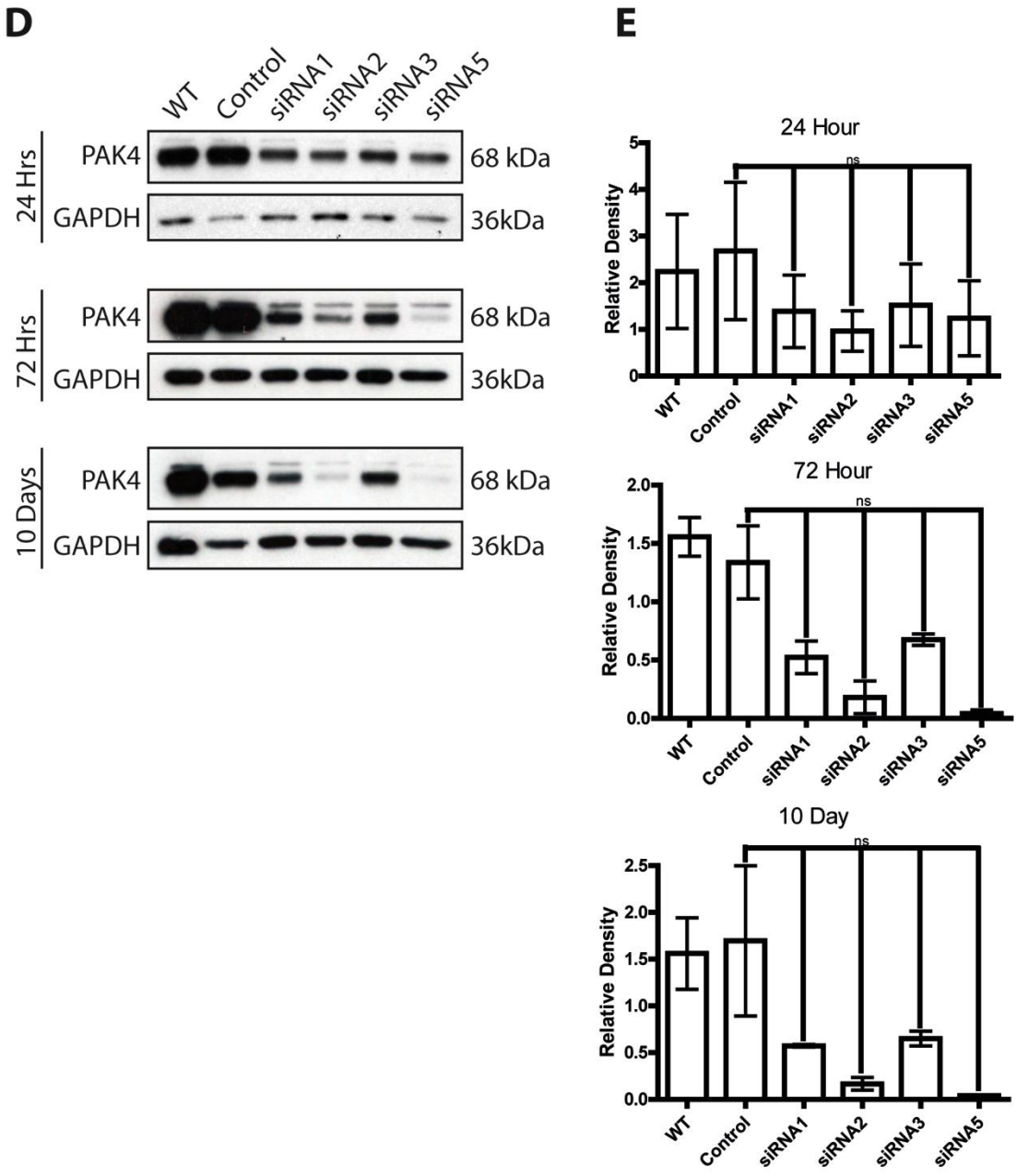


Figure 4.4: PAK4 knockdown in pancreatic cancer cell lines. In the first instance, a stable PAK4 knockdown cell line was intended to be made. However, despite a high transfection efficiency, no knockdown was evident (A). Using four siRNAs targeting PAK4 it was shown that knockdown of PAK4 was evident in both PaTu8988S (B) and PaTu8988T (D) cells after 24 hours. This effect lasted in excess of 10 days and it appeared to be greater with siRNA2 and siRNA5. Blots are representative of three independent experiments, which were quantified using densitometry (C and E), with each experimental repeat for each protein of interest being individually quantified using ImageJ and represented in graphical form after normalising to the loading control (GAPDH) * P<0.03.

After transfection, each cell line was lysed at 24 hours, 72 hours and 10 days (being trypsinised and reseeded as necessary to avoid cells becoming over confluent) and PAK4 expression investigated through western blotting for both PaTu8988S (**figure 4.4B** and **figure 4.4C**) and PaTu8988T (**figure 4.4D** and **figure 4.4E**). There was an obvious reduction in the expression of PAK4 with all four siRNA oligonucleotides even after only 24 hours. The expression reduced further after 72 hours and lasted in excess of 10 days. These results meant that the production of a stable cell line was not necessary in order to undertake assays where longer time frames were used. It was noted that out of the four siRNA oligonucleotides used, siRNA2 and siRNA5 lead to a greater level of PAK4 knockdown and so it was decided that moving forward that these two oligos would be used.

4.2.5 Depletion of PAK4 leads to a reduction in cell area and rounder cell shape in PaTu8988T cells

PAK4 was originally identified as a cytoskeletal regulatory protein (Abo et al., 1998), affecting both cell morphology and motility and filopodia formation (Callow et al., 2002). Thus, characterisation of cell area of each cell line after PAK4 depletion was performed. As PaTu8988S cells form tight colonies, making morphology studies challenging due to clustering, it was decided to look at cell morphology after PAK4 knockdown in the PaTu8988T cells. Control and PAK4 knockdown cells were seeded onto collagen coated coverslips (**figure 4.5A**). Following quantification (**figure 4.5B**), cells transfected with siRNA2 and siRNA5 were significantly smaller and rounder

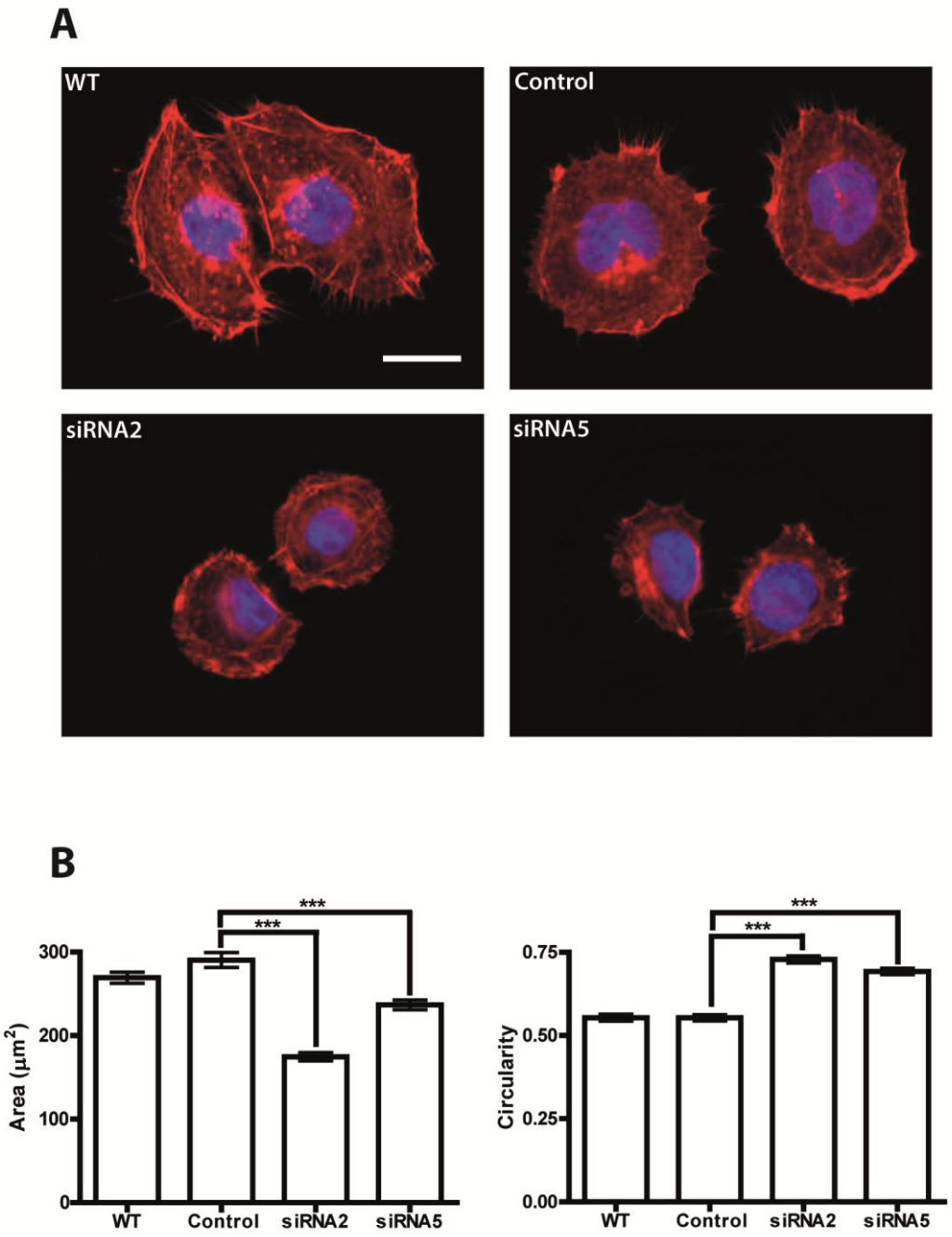


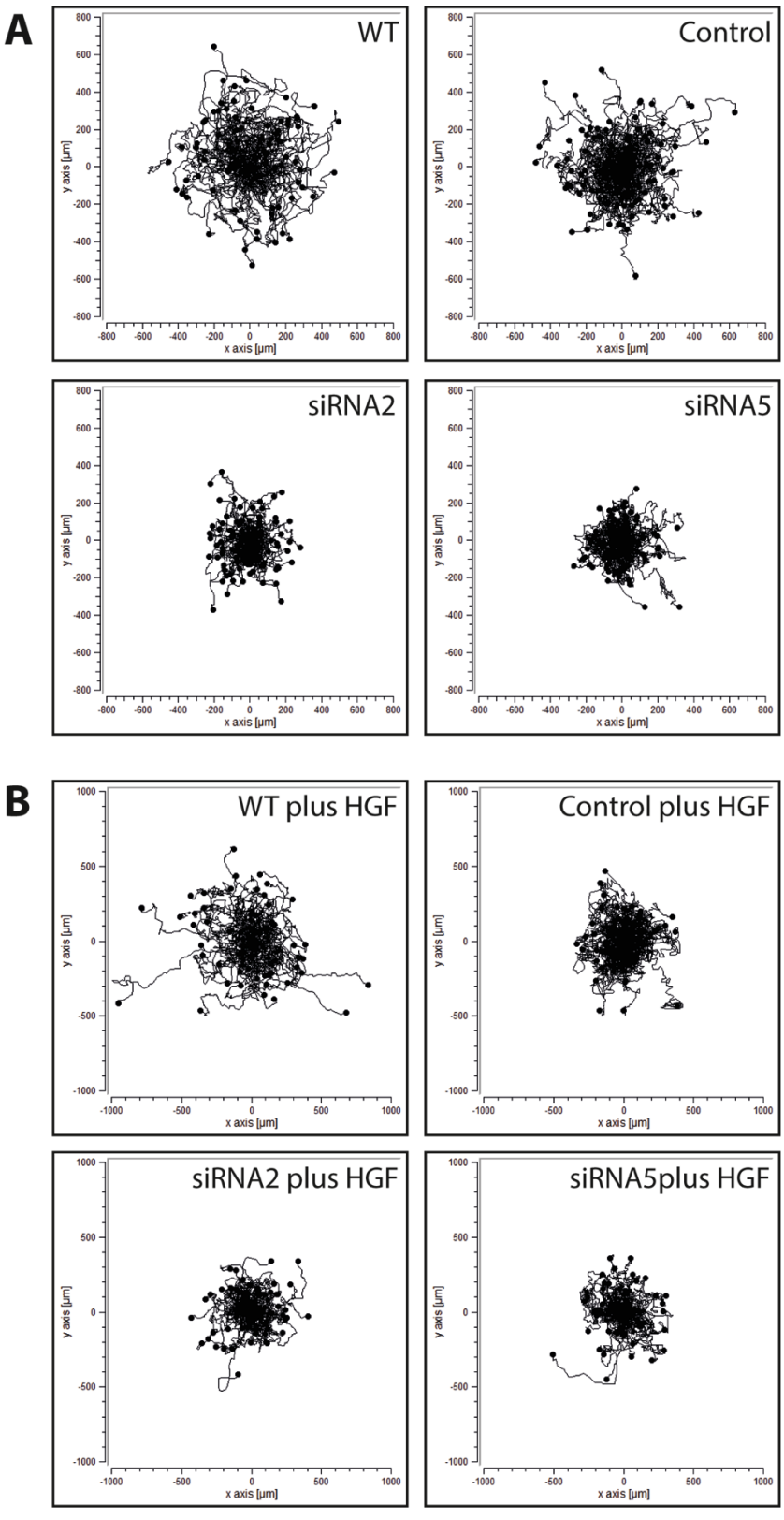
Figure 4.5: Effect of PAK4 depletion on PaTu8988T cell area. PaTu8988T cells were subjected to siRNA knockdown of PAK4 using two pre-validated siRNAs. In addition both a control siRNA and WT cells were used in morphological studies. Cells were reseeded onto collagen-coated coverslips and stained with DAPI (blue) and actin (red) (A). The cell area and circularity was then calculated using ImageJ and results quantified (B). There was a significant reduction in cell area and cells appeared rounder after PAK4 knockdown. Images are representative of three independent experiments. Scale bar = 10µm. Statistical significance was calculated using Student's *t*-test where *** $P < 0.0005$

than WT and control cells. These data were consistent with previous results observed in our laboratory using PC3 cells (Ahmed et al., 2008) and in HCT116 colon carcinoma cells (Tabusa et al., 2013).

4.2.6 Depletion of PAK4 reduces PaTu8988T cell migration

As stated, PAK4 is known to regulate actin cytoskeletal dynamics (Abo et al., 1998), through regulation of actin assembly and interacts with a plethora of proteins involved in cell migration including Cdc42, GEF-H1, LIMK and Gab1 (Ahmed et al., 2008; Callow et al., 2005; Paliouras et al., 2009; Wells et al., 2010). In particular, it has been shown that PAK4 interacts with LIMK in an HGF-dependent manner (Wells et al., 2002). Prostate cancer cells were also less responsive to HGF after PAK4 depletion. In addition, PAK4 has been shown to associate with Gab1 (a scaffold protein), relocalising to lamellipodia in response to HGF/c-Met signalling (Paliouras et al., 2009). Taken all together, the vast amount of research already published suggests that PAK4 plays a significant role in cell migration and metastasis. Although it has been shown that PAK4 knockdown leads to decreased pancreatic cancer cell migration (Kimmelman et al., 2008), little work has been done to look at the possible pathways PAK4 may lie in.

Having shown that PaTU8988T cells have a high migratory potential, which increased further after the addition of HGF (**figure 4.3**), control and PAK4 knockdown cells were tracked +/- HGF (**figure 4.6**). Over the course of three independent experiments, at least 60 cells per condition were tracked using


A
WT
Control
siRNA2
siRNA5
B
WT plus HGF
Control plus HGF
siRNA2 plus HGF
siRNA5plus HGF
y axis [µm]
x axis [µm]

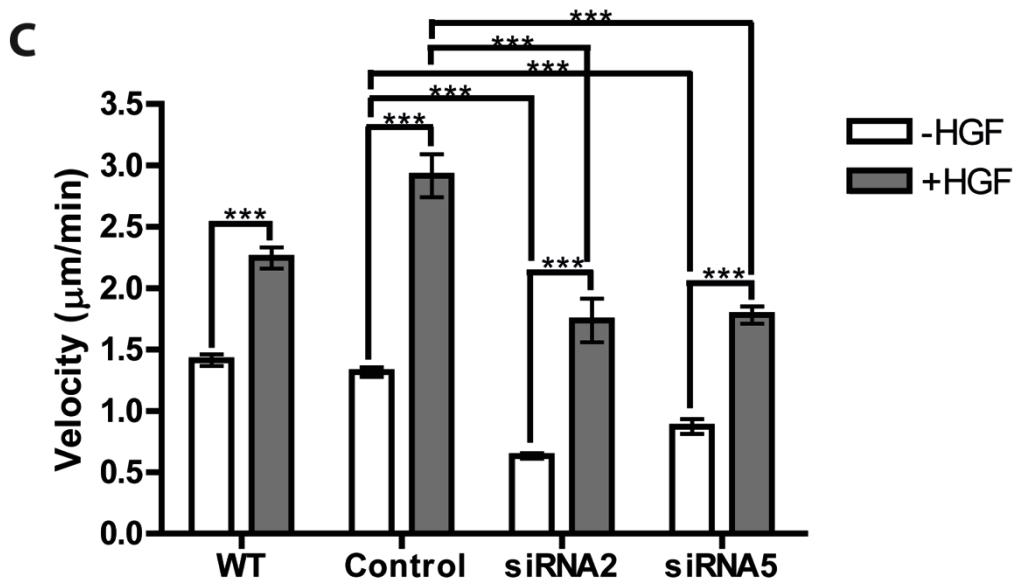


Figure 4.6 Effect of PAK4 knockdown on PaTu8988T cell migration. PaTu8988T cells were subjected to siRNA-mediated depletion of PAK4, reseeded on collagen and used within a random 2D migration assay both +/- the addition of HGF. In addition cells treated with an off-target control siRNA as well as WT cells were used as controls. Cells were imaged over the course of 12 hours, with an image taken at 5 minute intervals. Cells were then tracked using the ImageJ plugin MtrackJ and plot profiles produced for –HGF conditions (A) and +HGF (B) using the chemotaxis and migration tool from Ibidi. N=3 for each conditions tested and each data set was quantified (C). Results showed that upon depletion of PAK4 there was a significant decrease in PaTu8988T cell migration, which was evident with both siRNA2 and siRNA5. The same experiment was conducted with the addition of HGF, in order to determine whether PaTu8988T cells depleted of PAK4 had the same response to HGF as WT and control cells. As before, there was a significant difference in the average velocity of cells between PAK4 knockdown and control cells. However, cells had the same level of response to HGF. Statistical significance was calculated using Student's *t*-test where *** $P < 0.0005$

the ImageJ plugin MtrackJ and plot profiles produced using the Ibidi Chemotaxis and Migration tool (**figure 4.6A** and **figure 4.6B**). In the –HGF conditions, there was a visible change in migration rates and analysis revealed a significant reduction in mean velocity (**figure 4.6C**). Whereby cells treated with siRNA2 and siRNA5 lose their migratory capacity in comparison to both WT and control cells.

In addition to the –HGF independent experiments, where HGF had been added, as previous results showed, there was a significant increase in HGF induced cell migration in both the WT and control cells. Cells that had been depleted of PAK4 prior to HGF stimulation also had a significant increase in cell migration, although these were still significantly reduced in comparison to control cells. Previous work has demonstrated that cells were not as responsive to HGF after depletion of PAK4 (Ahmed et al., 2008) and although the PaTu8988T PAK4 knockdown cells responded to HGF it did not completely rescue the knockdown phenotype. PAK4 knockdown cells still had a significantly reduced migration rate in comparison to control cells, suggesting PAK4 is required for a full migratory response to HGF.

4.2.7 Production of a PAK4 RNAi rescue construct

As an additional control and validation method of the results observed through the use of the siRNA depletion of PAK4, a rescue construct was produced. Taking the target sequence of siRNA2 and using site-directed mutagenesis a GFP expressing siRNA resistant form of PAK4 was made. To ensure it was correct, PaTu8988T cells were subjected to knockdown and

transfection with the construct. Whole cell lysates were then produced and western blotting used to determine if the expression of the rescue construct was correct (**figure 4.7A**). After using the in house PAK4 antibody, a band at both 68 kDa and 95 kDa (PAK4 = 68 kDa + GFP = 27 kDa) can be seen.

Having shown that the construct successfully transfected into PaTu8988T cells, the rescue construct was used in random 2D migration assays. To perform the rescue experiment, 24 hours after depleting cells of PAK4 using siRNA, cells were transfected with the GFP tagged rescue construct before being filmed with both brightfield and GFP channels. Only GFP expressing cells were tracked. When comparing the migration velocity (-HGF), it was evident that cells expressing the GFP construct had a similar mean velocity to both WT and control cells with no significant difference being observed.

4.2.8 Reduced AKT phosphorylation in response to PAK4 knockdown

Having observed the phenotypic effect of PAK4 knockdown, the signalling mechanisms responsible for this were next investigated. It is known that PAK4 acts downstream of PI3K in epithelial cells (Paliouras et al., 2009; Wells et al., 2002) and that PI3K signalling is constitutively activated in pancreatic cancer (Bondar et al., 2002). Thus, the effect of PAK4 depletion on downstream effectors of the PI3K was investigated.

Previous results within this study have shown that PaTu8988T cells respond to HGF with an increase in the phosphorylation of AKT. Therefore the effect

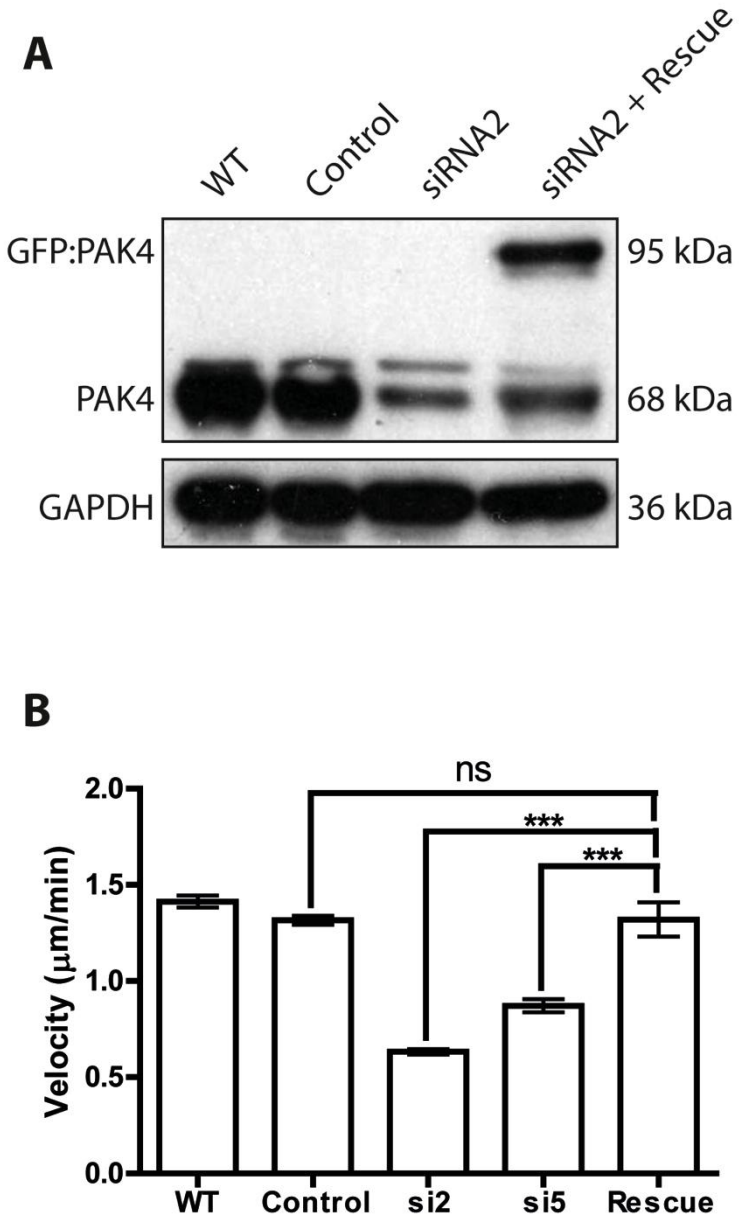


Figure 4.7: PAK4 siRNA rescue construct. Through site-directed mutagenesis, a PAK4 rescue construct was produced which was tagged with GFP. This was transfected into PaTu8988T cells after depleting cells of PAK4 using siRNA. Western blotting revealed that PaTu8988T cells expressed this rescue construct with a band being evident at 95 kDa (A). This rescue construct was then used in random 2D migration assays to see if the PAK4 knockdown phenotype could be rescued (experiments were not performed in the presence of HGF). Only GFP expressing cells were tracked and analysis revealved that there was a significant increase in migration after transfection with the rescue construct in comparison to knockdown cells. No significant difference was observed between rescue and control cells (B). Results from the rescue were gathered over 6 independent experiments and N=15 cells total due to technical difficulties. Statistical significance was calculated using Student's *t*-test where *** $P < 0.0005$

of PAK4 knockdown on the phosphorylation status of Akt was investigated. Whole cell lysates were made after PAK4 knockdown and Western blotting used to investigate the level of Akt phosphorylation (**figure 4.8A**). The results show that there is a correlation between the level of PAK4 and Akt phosphorylation, which is reduced as PAK4 is depleted. This correlates with newly published data in both NIH3T3 and gastric cancer cell lines (Fu et al., 2014; Gnad et al., 2013). The blot shown is representative of three independent experiments, which were quantified using densitometry (**figure 4.8B**).

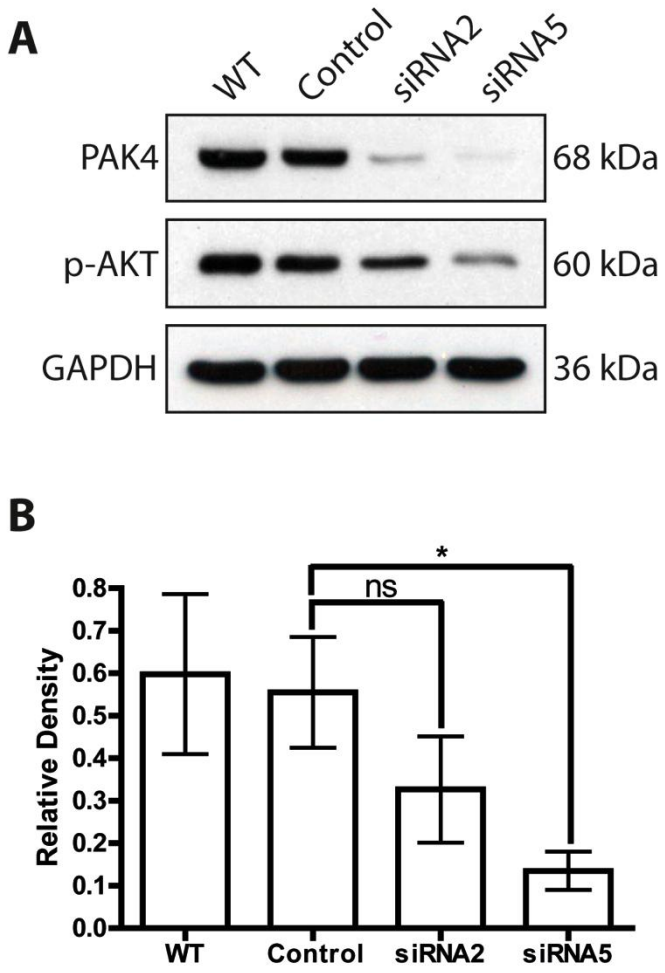


Figure 4.8: Reduction in AKT phosphorylation correlates with depletion of PAK4. The level of phosphorylation of potential downstream effector proteins of PAK4 was looked at after siRNA-mediated depletion of PAK4. Western blotting revealed that there was a correlation between PAK4 knockdown and the level of AKT phosphorylation (A). The results from three independent experiments were then quantified through densitometry (B), each experimental repeat was individually quantified using ImageJ and represented in graphical form after normalising to the loading control (GAPDH).

4.3 Discussion

In this chapter, the sister cell lines PaTu8988S and PaTu8988T were used to further interrogate the link between the PI3K pathway and PAK4 within pancreatic cancer. To do this, HGF stimulations were used in combination with siRNA knockdown of PAK4, in both morphological and migration assays.

Primarily the response of the cell lines to HGF was determined. As an output, both the phosphorylation of ERK and Akt were used, as both proteins are known to lie downstream of c-Met signalling in response to HGF (Tanahashi et al., 2013). Both PaTu8988S and PaTu8988T cells showed a significant increase in the phosphorylation of both of these proteins, with a response being evident after only 5 minutes. This confirms that HGF/c-Met signalling is highly active within these cell lines, reflecting the fact that it is upregulated within pancreatic cancer. With regards to PAK4, however, PaTu8988T cells showed no change in the phosphorylation status of this protein at Ser474 in response to HGF stimulation. As discussed, it was originally thought that phosphorylation at ser474 on PAK4 was a sign of its activation (Callow et al., 2002). However, with more recent literature suggesting that it is constitutively phosphorylated at this site (Baskaran et al., 2012; Ha et al., 2012) and knowing that PAK4 has been shown to be activated downstream of HGF we sought to confirm these results in the PaTu8988T cell line. The results shown here, demonstrate that PAK4 is likely to be constitutively phosphorylated at this site in this cell line, which is in line with the most current literature (Baskaran et al., 2012; Ha et al., 2012).

It was also shown that PaTu8988T cells had a significantly increased migratory response to HGF. It is known that both MAPK and PI3K/Akt pathways are activated downstream of HGF and c-Met, both of which can contribute to increased cellular motility. PaTu8988T cells are a highly invasive cell line and their migratory capacity was increased significantly after the addition of HGF. It is known that pancreatic cancer is a highly aggressive disease with for expression of c-Met and circulating levels of HGF being greatly increased (Kemik et al., 2009). It has been shown here the impact that these increased levels have on cellular migration, with a significant increase in PaTu8988T migration in response to HGF. In contrast to the PaTu8988T cells, PaTu8988S cells are known to be non-invasive (Marques et al., 2009) and it was shown within this chapter that they migrate very little, even after the addition of HGF. This is likely due to the high level of E-cadherin that they express at points of cell contacts, leading to tight colony formation, as shown in chapter three. Previous, published, work from our lab, which involved optimisation of a scatter assay using prostate cancer cell lines observed a similar result. HT29 cells were shown to form tighter colonies in comparison to DU145 cells. They did not exhibit the same scattering response to the same concentration of HGF and a higher concentration was required (Fram et al., 2014). It was therefore hypothesised that a higher concentration of HGF could be required to induce cell scattering in the PaTu8988S cell line. Despite have a relatively high expression of the c-Met receptor, it could also be speculated that the surface level may not be as high and thus preventing observation of a more migratory phenotype. However, as the PaTu8988T cell line showed both phosphorylation events

and an increased migratory capacity in response to HGF it was decided to pursue migration assays using this cell line.

It is known that PAK4 is upregulated in various cancer cell lines and tissue samples (Callow et al., 2002; Liu et al., 2008a; Siu et al., 2010a) and that it is oncogenic when overexpressed (Wong et al., 2013). Oncogenic PAK4 is also known to increase cell survival, motility and invasion (Kesanakurti et al., 2012; Siu et al., 2010a). Therefore, the effect of PAK4 knockdown in a pancreatic cancer setting was investigated. It was initially decided to produce a stable knockdown cell line using an already established shRNA vector (Whale et al., 2013). However, it was shown that this had little effect of the expression of PAK4 within the PaTu8988T cell line. This shRNA was a PAK4 specific pGIPz lentiviral vector, which uses a CMV promoter (a polymerase II promoter). It may be that an shRNA vector with a different promoter could prove more successful. U6 is a widely used polymerase III promoter and is known to be a strong member of the promoter class (Giering et al., 2008) and could therefore be a suitable alternative. It may also be of benefit to design a vector that targets a difference sequence within PAK4. However, for the purpose of this study, it was shown that siRNA provided an effective way to deplete both PaTu8988S and PaTu8988T cells of PAK4. A series of experiments, using a range of siRNAs from different companies in order to obtain optimal knockdown efficiencies, demonstrated that PAK4 levels were decreased after as little as 24 hours after siRNA treatment and lasted in excess of 10 days. This could indicate that once depleted, the recovery of PAK4 protein levels is difficult. It is known that PAK4 can translocate to the

nucleus, which is controlled by three nuclear export signals and two nuclear import signals. Within the nucleus it plays an important role in stabilising β-catenin by inhibiting its degradation (Li et al., 2012). As PAK4 contributes to the regulation of this protein, it could be hypothesised that it therefore contributes to its own regulation. This could provide some explanation as to the reason for the length of knockdown observed within this study, using siRNA. Two of the four siRNAs tested proved to be more successful in their knockdown of PAK4 and so only these were used in future experiments. However, also of note was the apparent increase in PAK4 expression in WT cells at later time points. This is most likely due to a confluency effect, where after being subjected to siRNA (control or PAK4) cells may have a slower rate of growth.

Further application of PAK4 siRNA demonstrated that with PaTu8988T cells, cells became smaller and rounder upon depletion of PAK4. This has previously been observed in our laboratory, where PC3 prostate cancer cells had a significant reduction in spread area after PAK4 knockdown (Ahmed et al., 2008). Another previous study showed that human pancreatic ductal cells expressing activated PAK4 became more elongated in comparison to control cells (Kimmelman et al., 2008). Therefore, it is likely the converse would be true for PAK4 depletion in pancreatic cancer cells overexpressing PAK4, as observed here. These observations could be considered to be due to the ability of PAK4 to affect actin cytoskeletal dynamics. It was also previously shown that PaTu8988T cells that were transfected with siRNA targeting PAK4 had a significant reduction in invasion in comparison to control cells

(Kimmelman et al., 2008). Within this study, random 2D migration assays on collagen were used to investigate the effect of PAK4 knockdown on PaTu8988T motility. The results were complimentary to the previous studies and demonstrated that depletion of PAK4 lead to significant decreases in cellular motility. Taken together, all these results demonstrate the necessity of PAK4 in pancreatic cancer cell migration. In addition, PaTu8988T cells that had siRNA depleted PAK4 were used in migration assays with the addition of HGF to investigate whether cells were less responsive to HGF as demonstrated previously in prostate cancer cells (Ahmed et al., 2008). Although PAK4 depleted cells still responded to HGF, they still had a reduced migratory capacity in comparison to control cells under the same conditions. These results suggest that PAK4 may be required to achieve a full response to HGF. However, the results may also infer that the reduced migratory capacity of PAK4 knockdown cells could be due to HGF-independent pathways, such as via K-ras/PI3K, and further investigation of these pathways would be required in order to make a definitive conclusion.

As an additional control, a siRNA rescue construct was designed and produced through site-directed mutagenesis. This was used in random 2D migration assays after siRNA mediated depletion of PAK4 and results gathered over six independent experiments. However, only a total number of 15 cells over the course of the experiments could be tracked. This was due to cell rounding/death and loss of expression during the time-lapse, with these cells being discounted. The other limitation of this experiment is that there is no way to test if the cells which are expressing the GFP tagged

rescue construct are ones which have also been targeted with siRNA, which is unknown with no visual cue. However, despite the limitations, the results did show that the use of the rescue construct was able to return cells in the PAK4 knockdown background to a normal migratory phenotype.

Having looked at PAK4 knockdown on morphology and migration, the effect of PAK4 knockdown on downstream effectors was investigated. Some preliminary work within our laboratory had suggested that Akt might be a downstream target of PAK4. Further investigation into the level of Akt phosphorylation after PAK4 knockdown was determined through western blotting. Results showed that there was a correlation between PAK4 depletion and the status of Akt phosphorylation. While this research was being carried out, new published data demonstrated that in NIH3T3 PAK4 knockout cells there was a reduction in Akt phosphorylation at Ser473 (Gnad et al., 2013) and the same has been observed after PAK4 knockdown in gastric cancer cell lines (Fu et al., 2014). These results confirm the observations in PAK4 depleted PaTu8988T cells. Taken together, these results provide evidence that PAK4 could be lying upstream of Akt.

Within this chapter, it has been determined that pancreatic cancer cell lines respond to HGF, leading to increased cell motility. It as also been demonstrated that PAK4 is essential for pancreatic cancer cell migration. It has also provided strong evidence that PAK4 lies upstream of Akt, with the phosphorylation of Akt being visibly reduced after depletion of PAK4, thus warranting further study of the link between PAK4 and the PI3K pathway.

4.4 Future Directions

One major concern with the results obtained during this chapter was the siRNA rescue construct. Due to cell death and loss of expression only a small number of cells could be tracked throughout the course of a number of experiments. Although the cells that were tracked did show that the PAK4 knockdown phenotype could be rescued, it would be worth formulating a more effective experiment to fully confirm these results. It would most likely be best to produce a stable knockdown cell line. This was the original experimental plan, however, the existing vector that had been successfully used within the laboratory previously (Whale et al., 2013) proved ineffective in knocking down PAK4 in the PaTu8988T cells. In order to continue with the experiments, it was decided to continue using only siRNA as the effects were long lasting. However, it may prove beneficial to use a vector using a different promoter to produce a stable PAK4 PaTu8988T cell line, which could potentially express a fluorescent protein. If then a rescue construct could be designed which was joined to a different fluorescent protein, cells co-expressing both vectors could be tracked and a true rescue phenotype could be observed. Having a stable cell line could also potentially help avoid as much cell death occurring as cells would undergo less stress and manipulation prior to the assay commencing.

Within this chapter, the effect of PAK4 knockdown on PaTu8988T cell migration was investigated both +/- the addition of HGF. Although there was a significant reduction in cell migration after depletion of PAK4 under both HGF conditions, there was still an increased migratory response in PAK4

knockdown cells after the addition of HGF. It was hypothesised, based on these results, that PAK4 may be required for a full migratory response to HGF. However, it could also be inferred from these results that an HGF independent pathway, such as PI3K, are required for PAK4-mediated migration. Further investigation would be required in order to determine the pathways required for PAK4-mediated cell migration. Although PAK4 has been shown to be activated downstream of HGF in epithelial cells (Wells et al., 2002), whether this is the same in pancreatic cancer is unknown. It would therefore be advisable to determine whether PAK4 is firstly activated downstream of HGF in these cell lines via the use of kinase assays. It would also be of interest to use chemotaxis assays such as a Boyden chamber or transwell migration assays to investigate a specific response to PAK4 in these cell lines before and after PAK4 knockdown. These would likely provide more conclusive evidence to support whether or not there was a definitive requirement for HGF in PAK4-mediated cell migration.

Although it was shown that PAK4 depletion led to a decrease in Akt phosphorylation the mechanism of PAK4 regulation on Akt has not been investigated within this study. There are a number of proteins that lie downstream of PI3K that can impact of Akt activity. For example, mTOR – specifically the mTORC2 complex – is a known regulator of Akt phosphorylation (Case et al., 2011; Moore et al., 2011; Tato et al., 2011). For full activation, Akt requires phosphorylation at Thr308 as well as Ser473. Phosphorylation at this site was not investigated as part of this study and it is known that this is mediated via PDK1 (Higuchi et al., 2008). This

demonstrates another mechanism through which the relationship between Akt phosphorylation and PAK4 could potentially be mediated via another protein. Therefore it would be beneficial to investigate whether a) PAK4 depletion affects Akt phosphorylation at Thr308 and b) whether the relationship between PAK4 and Akt is direct or mediated via other mechanisms/proteins.

Chapter 5
Results: Part Three
Investigation of the interaction of PAK4 with the PI3K pathway

Chapter 5: Investigation of the interaction of PAK4 with the PI3K pathway

5.1 Introduction

PAK4 activity is influenced by PI3K signalling (Fu et al., 2014; Wells et al., 2002) and more recent research has suggested that there are links between ras and PAK4 within pancreatic cancer (Chen et al., 2008; Kimmelman et al., 2008). This chapter sought to investigate these protein-protein interactions further, with the aims to clarify where PAK4 may lay within this Ras:PI3K pathway. It is known that the PI3K pathway can be activated either directly or indirectly by c-Met or downstream of Ras (Organ and Tsao, 2011). Therefore, whether PAK4 interacted with Ras or solely with PI3K was investigated.

Ras proteins are small GTPases, which are known to contribute significantly to oncogenesis. K-ras is one of the four ras isoforms (H-ras, N-ras, K-ras4A and K-ras4B), which are alternative splice variants of the RAS gene (Lowy and Willumsen, 1993; Plowman et al., 2003; Pylayeva-Gupta et al., 2011). They have a highly homologous domain structure except for a hypervariable region starting at amino acid 165. The N-terminus is identical in all four isoforms, with amino acids 32-40 constituting an effector-binding domain and provides a site for interactions with various effector proteins and allowing participation in a wide range of signalling pathways (Bar-Sagi, 2001; Castellano and Santos, 2011). Ras proteins are molecular switches and under normal cellular conditions, ras cycles between a GDP bound, inactive state and GTP bound, active state. The transition between these two states

is regulated via GEFs and GAPs (Biou and Cherfils, 2004; Colicelli, 2004; Malumbres and Barbacid, 2003; Vetter and Wittinghofer, 2001). However, K-ras is frequently mutated in pancreatic cancer, with the mutated form being observed in more than 90% of patient tumour samples. The most common mutation is on codon 12 to produce constitutively active K-ras (Collins and Pasca di Magliano, 2013; Zinsky et al., 2010). This leads to continual activation of downstream signalling pathways, including the PI3K pathway.

It is known that K-ras is able to directly activate PI3K. PI3K is a heterodimeric protein, consisting of two subunits; a catalytic subunit (P110) and a regulatory subunit (p85). It is the P110 subunit that is known to interact with ras via its RAS binding domain (RBD) and this is independent of p85 (Castellano et al., 2013; Gupta et al., 2007; Organ and Tsao, 2011). However, PI3K is also activated through association with receptors such as c-Met. This interaction can either be direct, whereby the p85 subunit associates with phosphorylated tyrosine residues via its SH2 domain, or indirectly via the scaffold protein Gab1 (Rocchi et al., 1998; Yu et al., 2001). After this association takes place, the catalytic P110 subunit is able to transfer phosphate groups to initiate downstream signalling.

PAKs have been shown to interact with the PI3K pathway. PAK4 activity has been shown to be dependent on PI3K, with pharmacological inhibition resulting in reduced PAK4 kinase activity (Wells et al., 2002). Group I PAKs also demonstrate links with PI3K, with a reduction in PAK1 activity being observed after inhibition of PI3K in colorectal cancer by LY294002,

downstream of gastrins (Huynh et al., 2014). Further to this, LY294002 mediated inhibition of PI3K in hepatocellular carcinoma cell lines also demonstrated reduced PAK1 activity (Xu et al., 2014). Contrary to results from the previous chapter where PAK4 was shown to act upstream of Akt, impacting on Akt phosphorylation, this study placed PAK1 downstream of Akt, with siRNA mediated depletion of Akt also reducing PAK1 activity (Xu et al., 2014). These highlight how different PAK isoforms can perform similar roles while resulting in differing outcomes.

As stated, pharmacological inhibition of PI3K through use of LY294002 has been shown to reduce PAK4 activity in both MDCK cells (Wells et al., 2002) and gastric cancer (Fu et al., 2014). However, this more recent study also observed that inhibition of PAK4 by PF-3758309 suppressed both PI3K/Akt and MEK/ERK signalling (Fu et al., 2014), suggesting these pathways are able to reciprocally activate each other.

Currently, there are no known binding partners of the proline rich regions within PAK4 (Dart and Wells, 2013). However, these proline residues provide putative binding sites for SH3 domain containing proteins (Dart and Wells, 2013). Previous work within our laboratory suggested that PAK4 could be interacting with the PI3K pathway through the regulatory subunit p85α, which was highlighted in a screen. However, this interaction wasn't fully validated and so the hypothesis that an interaction between PAK4 and the SH3 domain of p85α was investigated further within this chapter. In addition, it has been shown that Gab1 and PAK4 associate and colocalise within

lamellipodia, in response to HGF. This interaction was shown to be via the GID domain on PAK4 (Paliouras et al., 2009). Taken together, these results and preliminary data provide reasoning for further examination of the interplay of PAK4 with the PI3K pathway. There is strong evidence to suggest they could play a key role in pancreatic cancer progression and the results obtained throughout this next chapter sought to elucidate the nature of how PAK4 interacts with the PI3K pathway more completely.

5.2: Results

5.2.1 PAK4 interacts with K-ras

As part of the functional studies, the interaction of PAK4 with various components of the PI3K pathway was investigated. There have been links between K-ras and PAK4, showing PAK4 to be activated downstream of oncogenic K-ras (Chen et al., 2008; Choudhry et al., 2014; Yeo et al., 2014). HEK293 cells were transfected with GFP tagged K-ras and utilised in a glutathione-S transferase (GST) pulldown assay (**figure 5.1**). The results showed that there was a specific interaction between PAK4 and K-ras with no binding observed in the control lane.

5.2.2 PAK4 interacts with the p85α subunit of PI3K

Knowing that the PI3K pathway can be stimulated through c-Met and not only through K-ras (Organ and Tsao, 2011), the interaction between PAK4 and PI3K was next interrogated. It has also previously shown that the P110 subunit interacts with K-ras (Castellano et al., 2013; Gupta et al., 2007) and previous work in our laboratory suggests that there may have been an interaction between PAK4 and the regulatory subunit of PI3K, p85α. To investigate this, GFP tagged p85α was transfected into HEK293 cells and GST or GST tagged PAK4 truncated domain mutants were used to pulldown the p85α (**figure 5.2A**). It is known that PAK4 contains several proline rich regions and that these provide putative binding sites of SH3 domain containing proteins (Dart and Wells, 2013). Therefore, full length PAK4 and

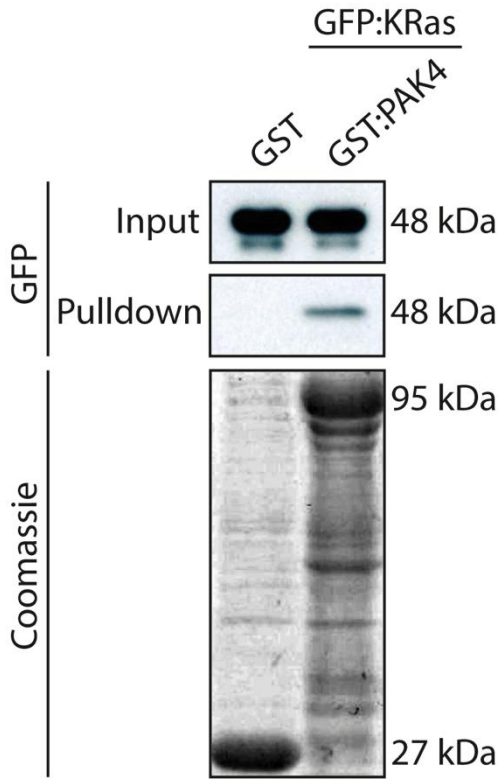


Figure 5.1: PAK4 interacts with K-ras. HEK293 cells were transfected with GFP:K-ras and subsequently used in a GST pulldown. GST and GST tagged PAK4 were used to pull down GFP:K-ras overexpressing HEK293 cells. Results demonstrated that there was a specific interaction between K-ras and PAK4, with no non-specific binding evident in the GST control lane. Blot shown is representative of three independent experiments. Coomassie shows the purified GST proteins.

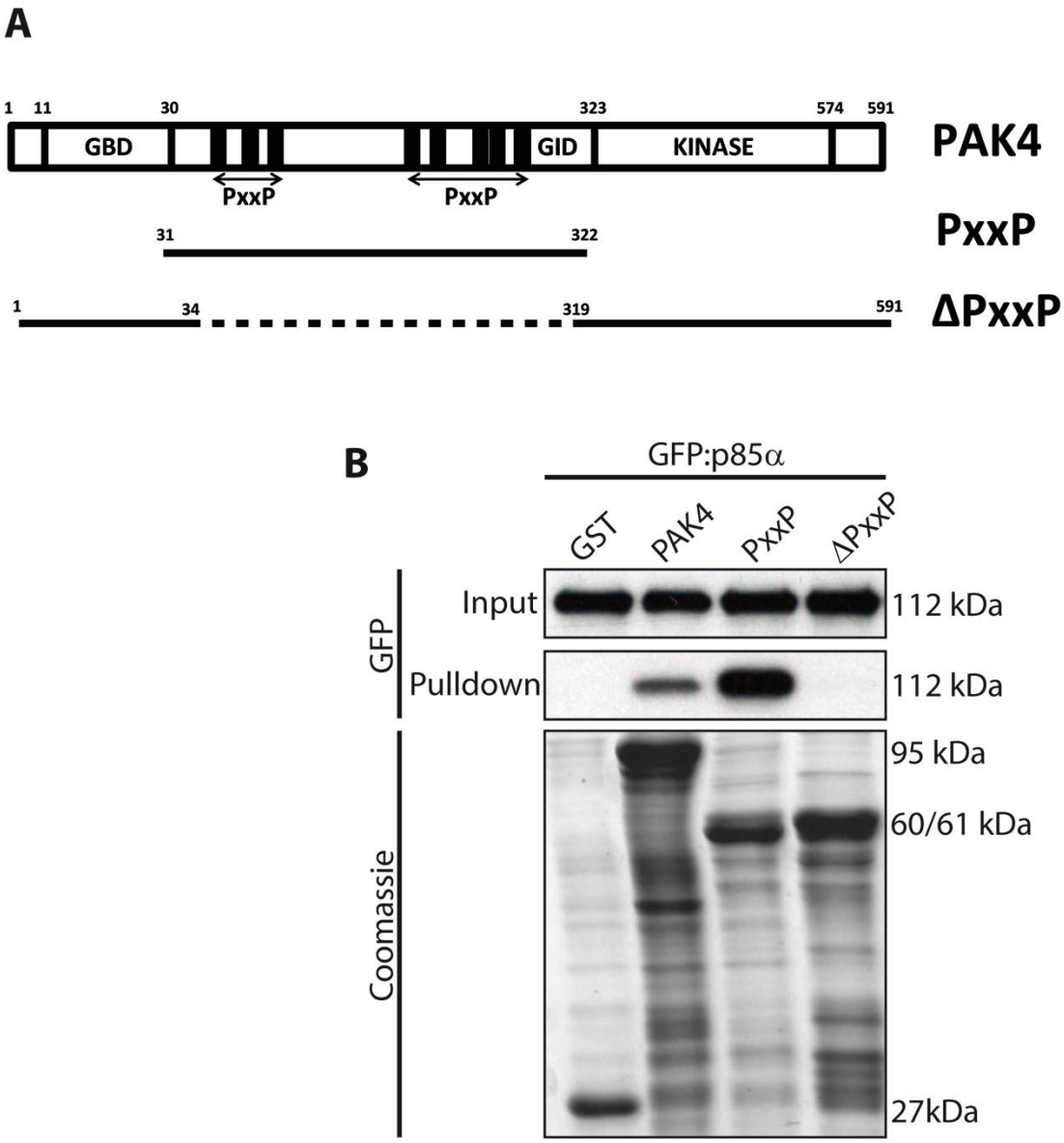


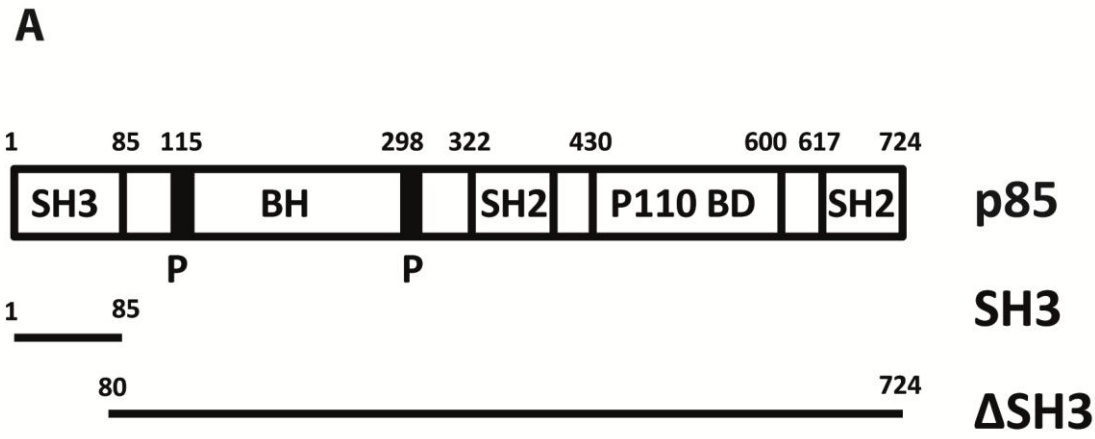
Figure 5.2: PAK4 interacts with the p85α subunit of PI3K. (A) Full length PAK4 domain structure (GBD: GTPase binding domain; PxxP: Pro-x-x-Pro amino acid sequence motifs; GID: GEF-H1/Gab1 interacting domain) and the amino acid regions that constitute the PxxP and ΔPxxP domain mutants of PAK4. (B) HEK293 cells were transfected with GFP tagged p85α and subsequently used in a GST pulldown assay. Domain mutants of PAK4 involving the PxxP region were used alongside GST (control) and full length wild-type PAK4. Results from the assay showed that there was a strong interaction between p85α and both PAK4 and the PxxP mutant. No binding was observed when the PxxP region had been deleted. These results demonstrated that the PxxP region is necessary for p85α binding. Results shown are representative of three independent experiments.

truncated domain mutants consisting of the proline rich region (PxxP comprising amino acids 31-322) and one with this region deleted (ΔPxxP comprising amino acids 1-34 plus 319-591) were produced (**figure 5.2A**).

These domain mutants would help determine whether p85α was binding within the proline rich region of PAK4, as hypothesised. The results from this GST pulldown assay revealed that there was binding between PAK4 and p85α and also between p85α and the PxxP region. These results were observed over three independent experiments. There was no observed binding between p85α and the ΔPxxP-truncated mutant (**figure 5.2B**). The results provide evidence for an interaction between p85α and PAK4, which is likely to be via the central proline rich region of PAK4.

5.2.3 Interaction between PAK4 and p85α requires SH3 domain

Having shown an interaction between PAK4 and p85α, with use of various domain mutants of PAK4 revealing the interaction to be within the PxxP region of PAK4, use of p85α domain mutants were used to further interrogate this interaction. It is known that the proline regions on PAK4 provide putative binding sites for SH3 domain containing proteins (Dart and Wells, 2013). It is also known that p85α contains an SH3 domain (Koyama et al., 1993). Therefore, it can be hypothesised that the interaction occurs between the PxxP region of PAK4 and the SH3 domain of p85α. In order to investigate this, two domain mutants of p85α were created (**figure 5.3A**). As well as full-length p85α, one consisting of just the SH3 domain (comprising amino acids


A
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85
115
298
322
430
600
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724
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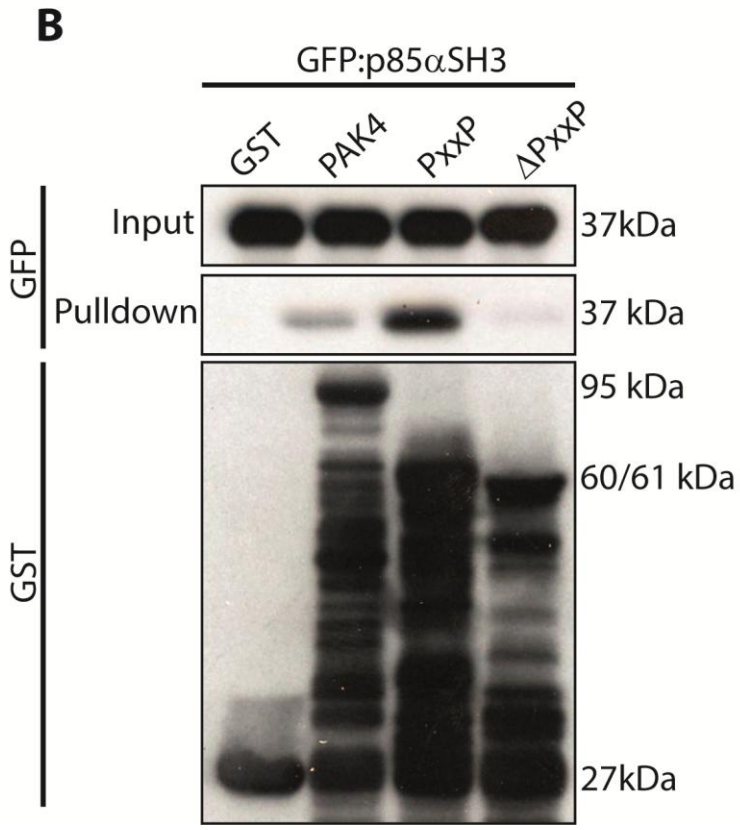
ΔSH3


B
GFP:p85αSH3
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37kDa
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95 kDa
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27kDa

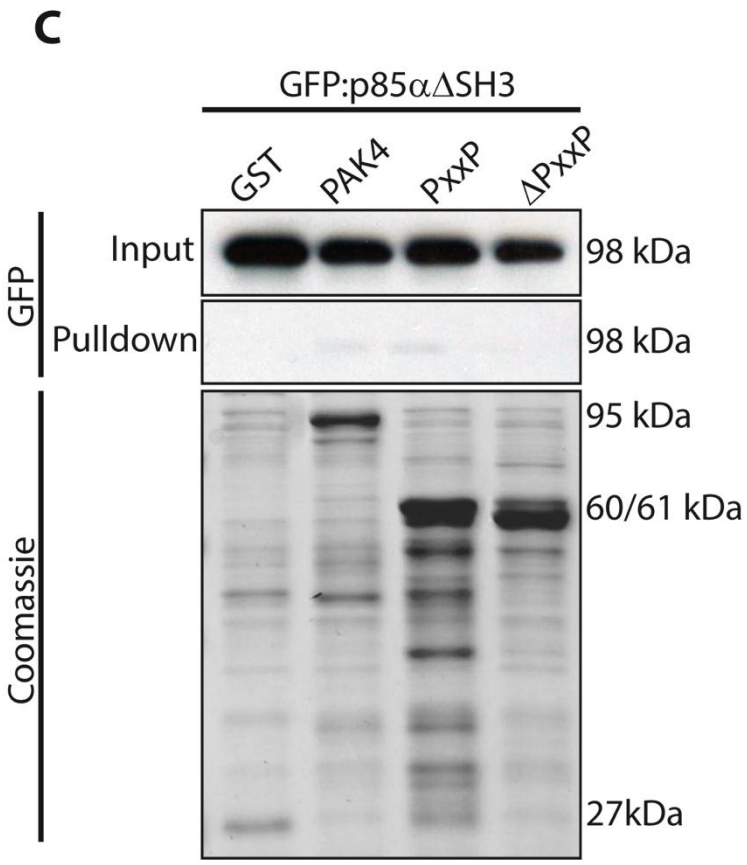


Figure 5.3: Requirement of SH3 domain of p85α for interaction with PAK4. Domain structure of p85 (SH3: Src homology 3 domain; P: Pro-rich region; BH: BCR homology domain; SH2: Src homology 2 domain; P110 BD: P110 binding domain). Two truncated p85α constructs were made; SH3 consisting of amino acids 1-85 and ΔSH3 consisting of amino acids 80-724 (A). These were both subsequently tagged with GFP. GFP:p85αSH3 was transfected into HEK293 cells and GST and GST tagged PAK4 derivatives used to pull down overexpressing cells (B). Western blotting revealed that p85αSH3 was able to bind to both PAK4 and PAK4PxxP with no binding observed between the SH3 domain and PAK4ΔPxxP. Conversely, when GST and GST tagged PAK4 derivatives were used to pull down HEK293 cells overexpressing GFP tagged p85αΔSH3, no binding was observed (C). For both assays, results shown are representative of three independent experiments.

1-85) and one missing the SH3 domain (ΔSH3, comprising amino acids 80-724) were created. These were tagged with GFP and transfected into HEK293 cells prior to their use in a GST pulldown. As before, in addition to GST, the three GST-tagged PAK4 derivatives were used to pulldown the GFP-tagged p85α truncation mutants and the results shown through Western blotting. The SH3 mutant was shown to bind to full length PAK4 and to the PxxP construct, but not to the ΔPxxP construct (**figure 5.3B**). This confirms the previous results, whereby it is most likely that PAK4 is interacting with the p85α via its SH3 domain. In contrast, when using the ΔSH3 construct, no binding could be observed between any of the PAK4 domain mutants (**figure 5.3C**). This further supports evidence for the SH3 domain being vital in the interaction between PAK4 and p85α.

5.2.4 Does p85α bind preferentially to one proline rich region of PAK4?

Within all PAK4 proteins, there are a variable number of core PxxP motifs, which are ligands for SH3 domain-containing proteins. However, this central PxxP-containing region differs in PAK4 in comparison to other PAK family members, containing three proline rich regions (Dart and Wells, 2013). However, previous to this study there were no known binding partners to these specific sites. Having demonstrated that p85α was able to bind within this PxxP region of PAK4, we sought to identify where the possible interaction was taking place. In order to do this, through site-directed mutagenesis, two full-length point mutation containing PAK4 constructs were produced (**figure 5.4A**). They contained a point mutation within different regions of the central, proline rich region of PAK4 (P105,108A and

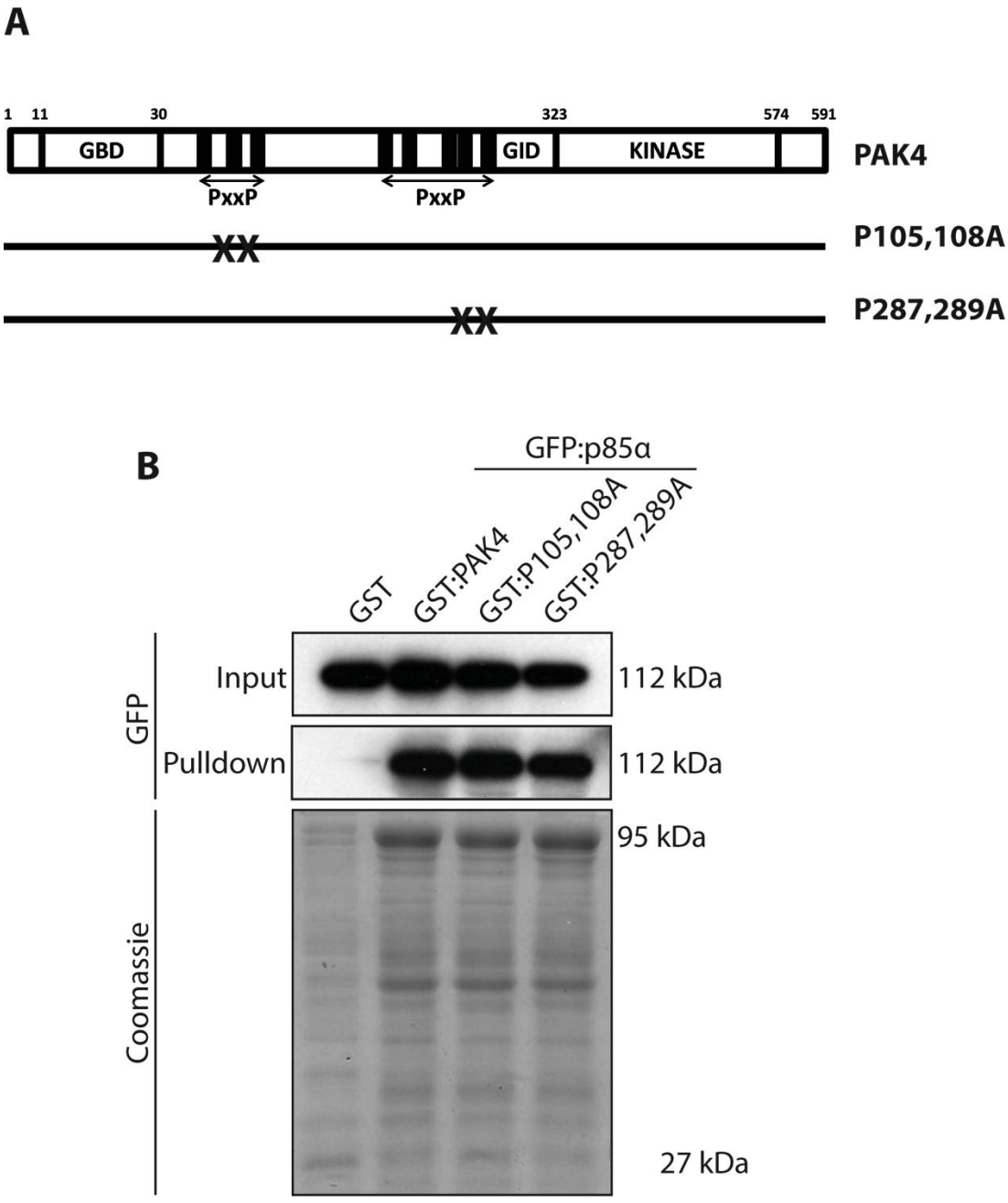


Figure 5.4: Point mutants of PAK4 reveal no preferential binding site within PxxP region. Through site-directed mutagenesis two full length PAK4 constructs containing point mutations at different sites within the PxxP region were produced (A). GST and the GST tagged PAK4 derivatives were used to pulldown HEK293 cells overexpressing p85α. Western blotting revealed there was no observed change in binding between any of the PAK4 constructs (B), suggesting no preferential binding site. Results shown are representative of three independent experiments.

P287,298A). These GST-tagged PAK4 point mutants and GST were used to pull down overexpressed GFP-p85α from transfected HEK293 cells. It was hypothesised that p85α would bind to one of these regions preferentially over the other which would be seen by decreased binding to one of the point mutants. However, the results showed that there was no change in p85α binding in either point mutant in comparison to full-length, wild-type PAK4 (**figure 5.4B**), suggesting no preference in binding site.

5.2.5 Interaction between PAK4 and p85α is dependent on Gab1

Having shown that there was no preferential binding within the proline rich region of PAK4 with p85α, more studies were used attempt to further elucidate the interaction between the two proteins. It is known that both PAK4 and p85α bind to the scaffold protein Gab1 (Abella et al., 2010; Paliouras et al., 2009), thus it was tested whether this protein was necessary for the two proteins to bind. Gab1 is an adapter protein that acts as a scaffold. It plays a critical role in a variety of signalling pathways, acting as a central site of multiprotein complex assembly. It is particularly known for acting downstream of c-Met in response to HGF. Activation of c-Met via HGF results in recruitment of Gab1 to the receptor and phosphorylation of specific tyrosine residues. This subsequently provides docking sites for various downstream signaling proteins including p85α (Nguyen et al., 1997; Ponzetto et al., 1994; Weidner et al., 1996). It was also more recently shown that Gab1 was able to bind to PAK4 via the GID domain (Paliouras et al., 2009). The interaction between PAK4 and p85α was therefore interrogated and a GST pulldown performed after cells were first treated with a siRNA pool for

Gab1. This would show whether Gab1 was necessary for the binding between PAK4 and p85α to occur. The assay showed that there was reduced binding between the two proteins after Gab1 had been depleted in the HEK293 cells prior to transfection with the p85α (**figure 5.5**), implicating a role for Gab1 in the interaction between PAK4 and p85α.

5.2.6 Use of PI3K inhibitor phenocopies PAK4 knockdown

It was speculated that inhibition of PI3K signaling would also impact on HGF-mediated responses, previously shown to be influenced by PAK4. LY294002 is known to be a potent inhibitor of PI3K and previous results from our laboratory demonstrated that an inhibition of PI3K using this compound lead to a decrease in PAK4 kinase activity (Wells et al., 2002). Therefore, further investigation into the effect of this PI3K inhibition on PAK4 was carried out in order to further establish the possibility of PAK4 lying within the PI3K pathway in pancreatic cancer.

The effect of using the inhibitor on Akt phosphorylation in the PaTu8988T cells was first determined (**figure 5.6A**). PaTu8988T cells were subjected to treatment either with 20μM LY294002 or with DMSO as a control. Lysates were made from cells in full growth media, starve (0% FBS) media and those that had been stimulated with HGF and the level of Akt phosphorylation determined through western blotting (**figure 5.6A**). The results showed that there was complete abolition of Akt phosphorylation in all conditions after addition of the LY294002. This demonstrates that LY294002 is a potent inhibitor of PI3K activity within these pancreatic cancer cells. Subsequently

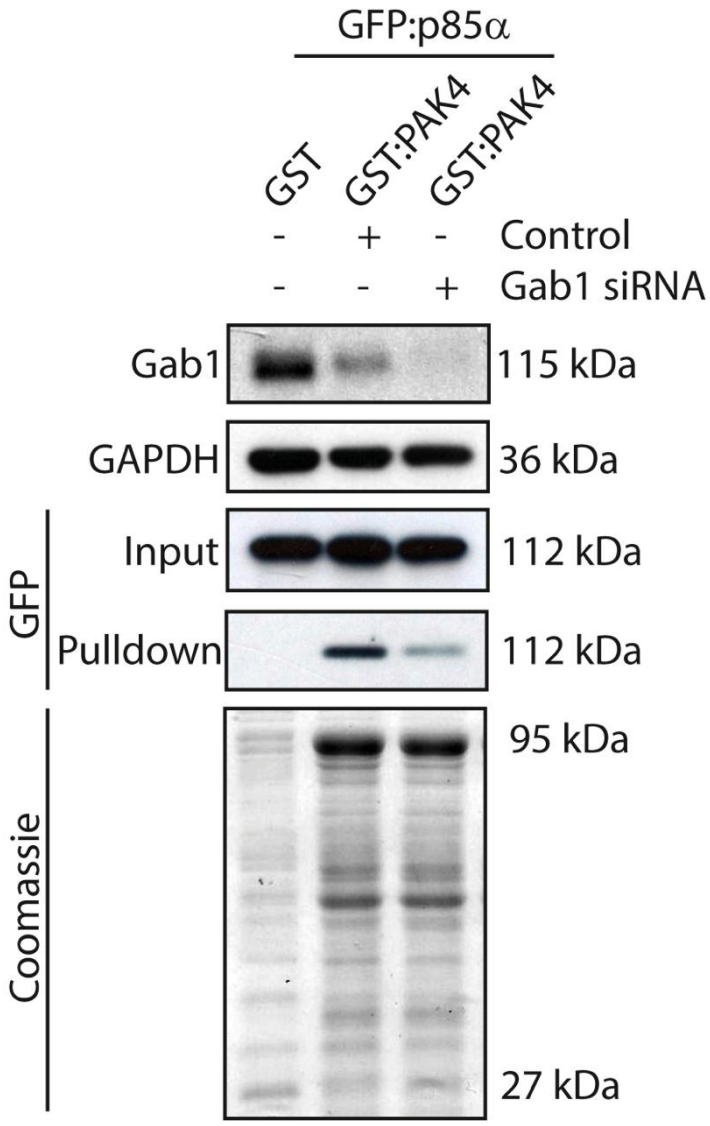


Figure 5.5: The interaction between PAK4 and p85α is dependent on Gab1. HEK293 cells were subject to siRNA mediated knockdown on Gab1. Subsequently they were transfected with GFP tagged p85α. GST and GST tagged PAK4 were used to pull down p85α overexpressing HEK293 cells. Western blotting revealed a reduction in binding between PAK4 and p85α after Gab1 knockdown. Results shown are representative of three independent experiments.

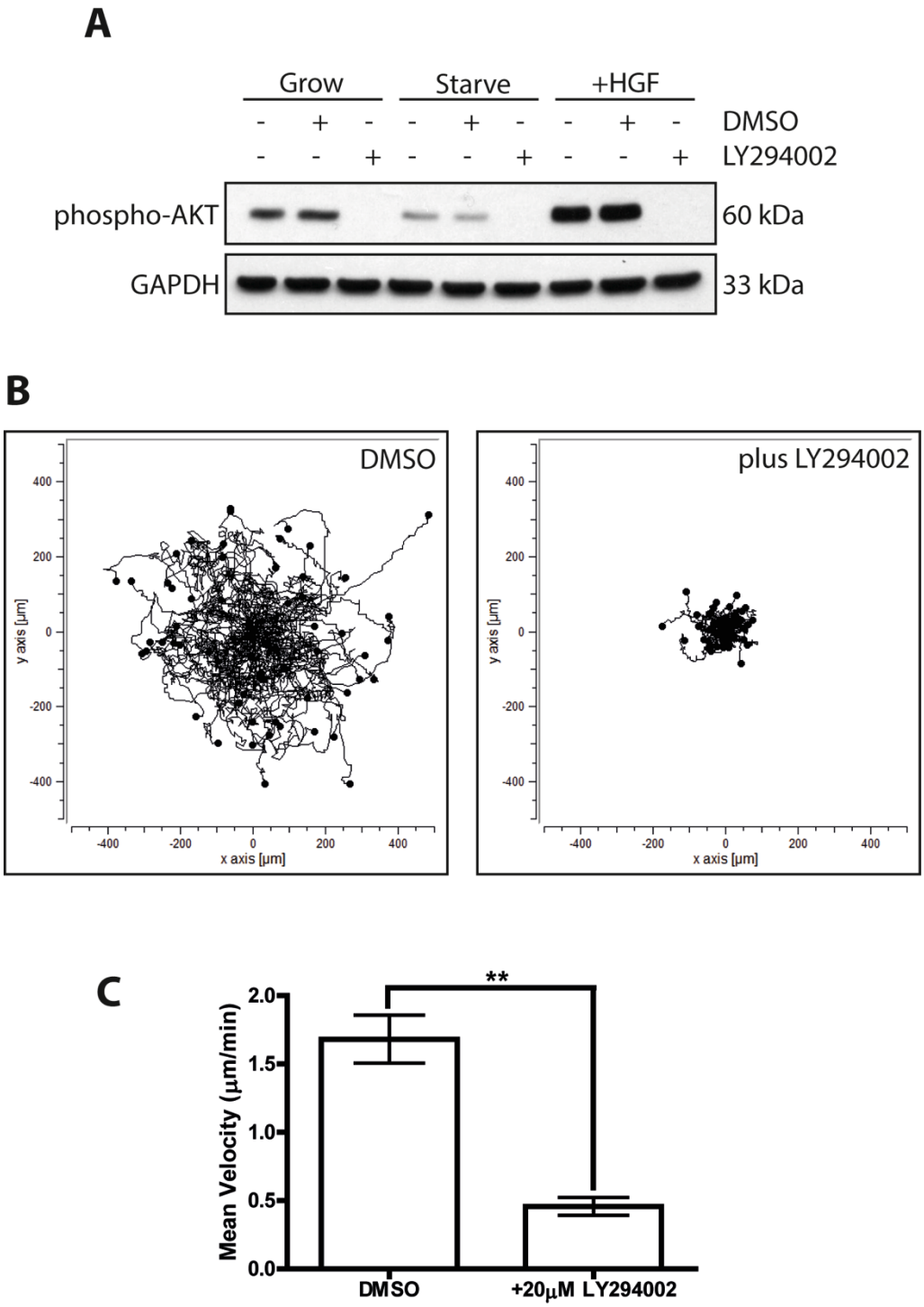

A
Grow
Starve
+HGF
- + - - + - - + -
DMSO
- - + - - + - - +
LY294002
phospho-AKT
60 kDa
GAPDH
33 kDa
B
DMSO
plus LY294002
y axis [µm]
x axis [µm]
400
200
0
-200
-400
C
**
Mean Velocity (µm/min)
2.0
1.5
1.0
0.5
0.0
DMSO
+20µM LY294002

Figure 5.6: Effect of using the PI3K inhibitor LY294002. (A) The effect of using 20μM LY294002 on AKT phosphorylation in PaTu889T cells was investigated in grow, starve and after the addition of HGF. In all three conditions, upon exposure to the inhibitor there was complete ablation of phosphorylation of AKT at Ser473. The migration of PaTu8988T cells was also studied after addition of LY294002. Cells were tracked for 12 hours with an image being captured at 5-minute intervals. Cells were tracked using the ImageJ plugin MtrackJ and plot profiles produced (B) using the chemotaxis and migration tool from Ibidi. Results were analysed and showed that there was a significant reduction in migration upon exposure to the inhibitor (C). Statistical significance was calculated using Student's *t*-test where ** $P < 0.005$

the effect of adding LY294002 to PaTu8988T cells prior to a random 2D migration assay was investigated. As expected, there was a significant reduction in cell migration; with cells being tracked using ImageJ and trajectory plots produced using the chemotaxis and migration tool from Ibidi (**figure 5.6B**). The mean velocity was calculated and graphed (**figure 5.6C**) and cells migrated very little after the addition of the inhibitor.

5.3 Discussion

In this chapter, multiple GST pulldown assays were performed in order to investigate the potential relationship PAK4 has with the Ras:PI3K pathway. Various mutants of both PAK4 and p85α were created to analyse the possible domain interactions and the effect of pharmacological inhibition of PI3K was also investigated.

Previous research has demonstrated a link between K-ras and PAK4 within pancreatic cancer (Chen et al., 2008; Kimmelman et al., 2008), and this interaction was investigated further within this chapter. K-ras is important in the initiation of the PI3K pathway, but it has also been shown within colon cancer cell lines, that both PAK4 and PAK1 can impact on K-ras signalling independent of this pathway (Tabusa et al., 2013). However, more recently published data has shown that in both PAK4 knockout NIH3T3 cells and siRNA mediated depletion of PAK4 in gastric cancer cells lines leads to a reduction in AKT phosphorylation downstream of PI3K (Fu et al., 2014; Gnad et al., 2013). Furthermore, in the observed phenotype in the PAK4 knockout cells could be rescued through introduction of constitutively active K-ras but not Cdc42 (Gnad et al., 2013), providing additional evidence to support the hypothesis that PAK4 is involved within this signalling pathway.

Therefore, it was important to further elucidate the relationship between K-ras and PAK4. Pulldown assays, where full length K-ras and PAK4 were used, demonstrated that there was binding between the two proteins. These results establish a K-ras/PAK4 relationship. However, the PI3K pathway can

also be activated independent of K-ras activity (Castellano and Santos, 2011). It was therefore necessary to investigate further the relationship of PAK4 with components further down the signaling pathway.

Taking into consideration preliminary data from our laboratory that suggested a potential interaction between the regulatory p85α subunit of PI3K with PAK4, initially, GST pulldown assays were conducted using the full-length constructs of both proteins. It was also hypothesised that binding between p85α and PAK4 could be within the proline rich region of PAK4. To date, no known interacting partners have been identified, but it is a logical assumption that the interaction between p85α and PAK4 involves this region. It is known that these proline residues provide putative binding sites for SH3 domain-containing proteins (Dart and Wells, 2013) and that p85α contains an N-terminal SH3 domain. Therefore two truncated domain mutants of PAK4 were created; PxxP, which comprised the proline region only and ΔPxxP, that had had this region deleted. These initial studies revealed that there was an interaction between PAK4 and p85α and that this appeared amplified when only the PxxP region was used within the pulldown. Confirming the hypothesis that the proline residues were required for the interaction to occur, no binding was evident in the deletion mutant.

Having determined that there was an interaction between the PxxP region of PAK4 and p85α, it was sought to confirm which region of p85α was binding. As aforementioned, the proline residues provide binding sites for SH3 domains and it was therefore hypothesised that the interaction was occurring

between the PxxP region of PAK4 and the SH3 domain of p85α. In order to confirm this, two domain mutants of p85α were produced, tagged with GFP. These were the SH3 domain alone and a construct with the N-terminal SH3 domain deleted. As predicted, there was binding of the SH3 domain to the PxxP region of PAK4 and no binding observed after deletion of this domain. These data provide strong evidence for this novel interaction between PAK4 and p85α and heavily implicate the involvement of PAK4 in PI3K signalling.

To further investigate this interaction, two point mutants of PAK4 were created through site-directed mutagenesis. These were used to answer whether p85α was binding preferentially to one of the proline rich binding sites of PAK4. However, the pulldown assay revealed no change in binding between either point mutant and wild-type PAK4. Taking these results into consideration, the domain mutants of PAK4 were looked at in more detail. It was noted that the PxxP mutant was composed of amino acids 31-322, which not only encompasses this proline rich region, but also the majority of the GID.

It is known that the GID of PAK4 is the site of Gab1 interaction (Paliouras et al., 2009) and also that Gab1 is a known binding partner of p85α (Abella et al., 2010). It was therefore thought that the interaction between PAK4 and p85α may be dependent on the scaffold protein Gab1, with it either being involved in a cooperative binding complex or acting as a scaffold. Knockdown of Gab1 prior to performing a pulldown revealed that there was indeed a reduction in binding after Gab1 had been depleted. These results

suggest that Gab1 is therefore necessary for the interaction between PAK4 and p85α.

However, previous results obtained showed that there was an interaction between the SH3 domain of p85α and the PxxP region of PAK4. It is known that p85α binds to Gab1 via its SH2 domain (Rocchi et al., 1998; Yu et al., 2001). Therefore, if Gab1 was necessary for binding between p85α and PAK4 how was there an interaction present when the SH2 domain required for Gab1 binding was deleted? In response to this issue, it could be that PAK4 binds directly to the SH3 domain of p85 via proline residues within the PxxP region but also indirectly via Gab1 which p85 binds to via its SH2 domain. This could go some way to explain as to why there is reduced binding after Gab1 knockdown but also why there is binding between just the SH3 domain and the truncated PxxP PAK4 construct.

More recently, the mode of PAK4 activation has been under examination. One study has identified a pseudosubstrate containing a specific sequence (R^{49}PKP) within the N-terminal of PAK4, which binds to the kinase domain and results in constitutive autoinhibition. They concluded that this sequence was necessary and sufficient to inhibit PAK4 kinase activity, but also that there were potentially other regions that may facilitate complete inhibition (Ha et al., 2012). Within this same study, they found that Src SH3, but not β-PIX SH3, was able to activate PAK4 (Ha et al., 2012). It has been demonstrated that the tertiary structure of the binding sites in the p85α and Src SH3 domains are similar (Koyama et al., 1993) and these two proteins have been

observed to have the same substrate in other studies (Shen et al., 1999). It could therefore be hypothesised that upon binding to PAK4 that the SH3 domain of p85α could provide a mechanism to relieve PAK4 autoinhibition.

As discussed, the use of a known PI3K inhibitor (LY294002) has been shown to impact of PAK4 kinase activity (Wells et al., 2002). To compliment the biochemical studies, it was decided to investigate as to whether this pharmacological inhibition of PI3K phenocopied the results observed in the previous chapter. After PAK4 knockdown there had been a significant decrease in cancer cell motility and after PaTu8988T cells had been pre-incubated with LY294002, there was also a reduction in cell migration, albeit to a greater extent. However, taken together, these results demonstrate the requirement for both PAK4 and PI3K signalling within pancreatic cancer cell migration.

Within this chapter, a novel interaction between PAK4 and p85α has been shown. Although the exact nature of this interaction needs further investigation, it may be plausible to hypothesise, based on the results gathered, that there is a direct interaction between the SH3 domain of p85α and the PxxP region of PAK4 but also an indirect interaction via Gab1.

5.4 Future Directions

Many protein-protein interactions are transient. It is known that PAK4 is involved in many different signalling pathways and so looking at interacting partners at an endogenous level can be difficult. Within this chapter, GST pulldown assays were used to successfully identify a number of new PAK4-interacting proteins. However, there were limitations, as discussed in **section 5.3**. Due to time constraints, more in depth analysis could not be completed, however, there are a number of ways in which these interactions could be further investigated.

Although p85α was newly identified as a PAK4 binding partner, the full nature of this interaction requires further interrogation. The PAK4 PxxP domain mutant was found to contain the GID, the Gab1 interaction domain. Therefore, whether Gab1 is providing a scaffold between PAK4 and p85α hasn't fully been answered. It would therefore be necessary to produce a PxxP domain mutant that had the GID entirely deleted. It would also be of benefit to produce a construct that had the GID intact, but with the PxxP region deleted, and use this to determine if p85α could still be detected. It could also be considered of use to look at binding between p85α and Gab1 after PAK4 knockdown and investigate whether this affected this known interaction. These experiments would provide further insight into the interplay between these three proteins.

However, ultimately it would be ideal to determine if the interaction between PAK4 and p85α was direct as well. This is the limitation with pulldown

assays; although they are able to show an interaction, they do not confirm is this is direct of indirect. One relatively simple technique, which could be used to determine whether there was a direct interaction, would be a far Western blot. This is a direct binding assay whereby interacting partners can be identified within samples. As opposed to the use of an antibody, purified proteins are incubated with the nitrocellulose membrane after SDS-PAGE and electroblotting (Johansson et al., 2004). Another method by which to study direct protein-protein interactions is the yeast two-hybrid system. Within this system, the protein of interest is fused to the DNA binding domain (bait). The interacting protein is then fused to the activation domain (prey). In short, if the two proteins interact, it allows transcription of the reporter gene which can be subsequently detected (Bruckner et al., 2009). Ultimately, it would be highly beneficial to determine whether a direct interaction between PAK4 and p85α is present.

Also within this chapter it was demonstrated that the SH3 domain of p85α is able to bind to PAK4. It was hypothesised that this could ultimately lead to increased PAK4 kinase activity through relief of PAK4 autoinhibition. It would therefore be advisable to perform kinase assays to test this theory.

Chapter 6
Results: Part Four
An organotypic model of pancreatic cancer

Chapter 6: An organotypic model of pancreatic cancer

6.1 Introduction

One of the hallmarks of pancreatic cancer is the formation of fibrotic tissue; an event often referred to as the desmoplastic reaction, which is perhaps one of the most prominent stromal reactions of all epithelial tumours (Vonlaufen et al., 2008b). This tissue is composed of various extracellular matrix proteins including collagens and matrix metalloproteinases, an increase in myofibroblastic pancreatic stromal cells and the presence of immune cells (Bardeesy and DePinho, 2002; Pandol et al., 2009; Schneiderhan et al., 2007). Stromal cells including pancreatic stellate cells and cancer-associated fibroblasts contribute to tumour development and progression through cross talk and communication with cancer cells via a number of signaling pathways including Hedgehog, Notch and TGFβ (Bailey and Leach, 2012). The release of various growth factors also contributes to the formation of fibrotic tissue, with the activity of these stellate cells meaning that the rate of extracellular matrix deposition is exceeding that of ECM degradation (Phillips, 2012). Often the desmoplastic stroma can contribute between 70-90% of the actual tumour volume (Erkan et al., 2012a; Hwang et al., 2008).

This characteristic feature of pancreatic cancer and pronounced desmoplastic tissue is characterised by dense bundles of collagen, loss of basement membrane integrity and invasion of malignant cells into the interstitial matrix (Apte et al., 2004; Mahadevan and Von Hoff, 2007). It has also been shown to contribute to tumour aggressiveness, with pancreatic stellate cells promoting proliferation, migration and invasion of pancreatic

cancer cells (Bailey and Leach, 2012; Hwang et al., 2008; Vonlaufen et al., 2008a). It has also been shown *in vivo* that nude mice injected with a combination of cancer and stellate cells had a higher frequency of tumour formation, increased tumour volume, proliferation and desmoplasia in comparison to those injected with only cancer cells (Bachem et al., 2005; Schneiderhan et al., 2007; Vonlaufen et al., 2008a). It is also well known that the exclusive microenvironment observed in pancreatic cancer contributes to chemoresistance, due to the reduction in drug penetration (McCarroll et al., 2014; Michl and Gress, 2012; Whatcott et al., 2012).

Taken together, this highlights that the tumour microenvironment plays a significant role in disease progression and influences heavily in pancreatic cancer. Therefore research into this complex disease needs to take this into consideration. 3D organotypic models are able to bridge the gap between simple 2D *in vitro* assays and far more complex *in vivo* models. Organotypic models are invaluable tools in which to study cancer development and progression as they recapitulate the features of the disease and better mirror the *in vivo* environment. They are also more easily manipulated to meet the specific requirements of the assay, depending on the exact features under scrutiny.

Research has implicated the HGF/C-met signaling axis in promoting the desmoplastic reaction (Mahadevan and Von Hoff, 2007) and squamous cell carcinoma cells that had been depleted of PAK4 have shown reduced invasion in a 3D organotypic invasion assay (Zanivan et al., 2013). Within

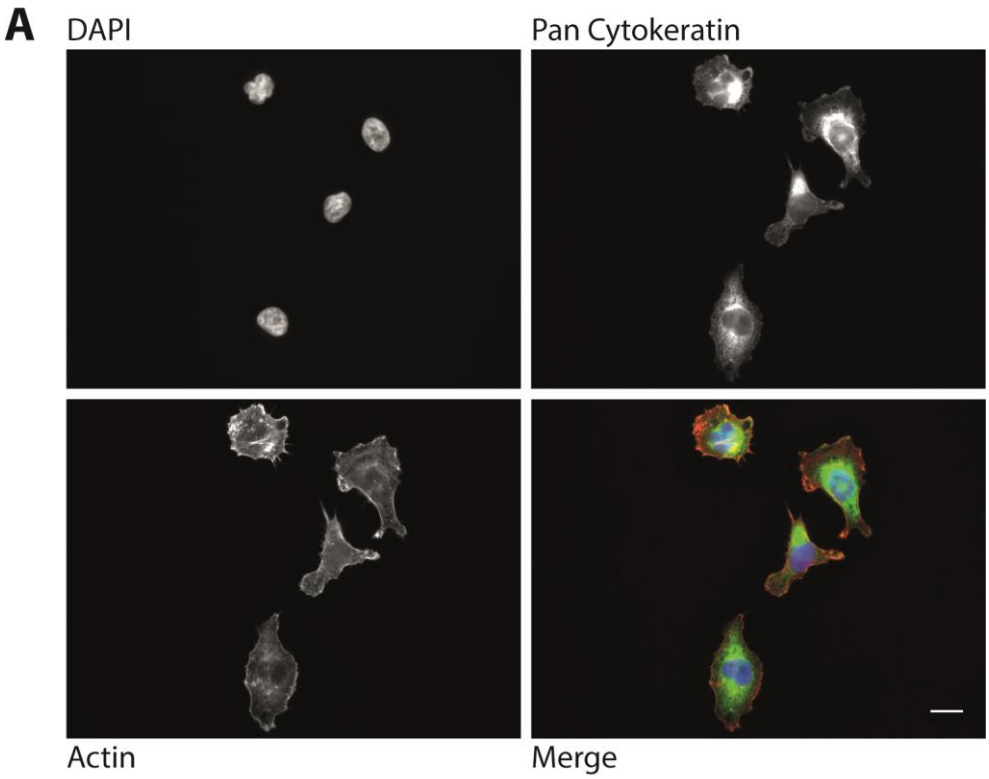
this final results chapter, an organotypic model of pancreatic cancer was used to study the invasion of pancreatic cancer cells after PAK4 knockdown and whether this was phenocopied by cells subjected to pharmacological inhibition of PI3K. This would enable examination of the role PAK4 plays in pancreatic cancer invasion in an environment that better simulates conditions found *in vivo*.

6.2 Results

6.2.1 Distinguishing between pancreatic stellate and cancer cells

Within the organotypic model, it is important to be able to distinguish between the stellate cells and the cancer cells being used. This is in order to determine specifically cancer cell invasion. The PS-1 cells are a pancreatic stellate cell line, which were isolated, immortalised and characterised previously (Froeling et al., 2009). Stellate cells were already known to contribute to fibrosis during chronic pancreatitis (Masamune et al., 2009); however, they were identified as producers of ECM proteins that are major contributors to pancreatic tumour stroma (Apte et al., 2004). Several pancreatic stellate cell selective markers including desmin, GFAP and nestin as well as α-smooth muscle actin (as a marker of activation) have been identified (Apte et al., 2004; Apte et al., 2013). Knowing that activated stellate cells are present in the tumour stroma and contribute heavily to growth factor and matrix protein production involved in pancreatic cancer progression, α-smooth muscle actin was chosen as a suitable marker to distinguish these cells from the cancer cells used. It is also known that adenocarcinomas of the pancreas express cytokeratins and are used to identify cancer cells.

To ensure that these markers were suitable for use within the organotypic model, both PaTu8988T and PS-1 cells were seeded onto collagen-coated coverslips and stained for these marker using specific antibodies. It was shown that PaTu8988T expressed cytokeratin (**figure 6.1A**) whereas the PS-1 cells had no expression evident (**figure 6.1B**). Conversely, PaTu8988T cells had no α-SMA expression (**figure 6.1C**), in comparison to the PS-1


A
DAPI
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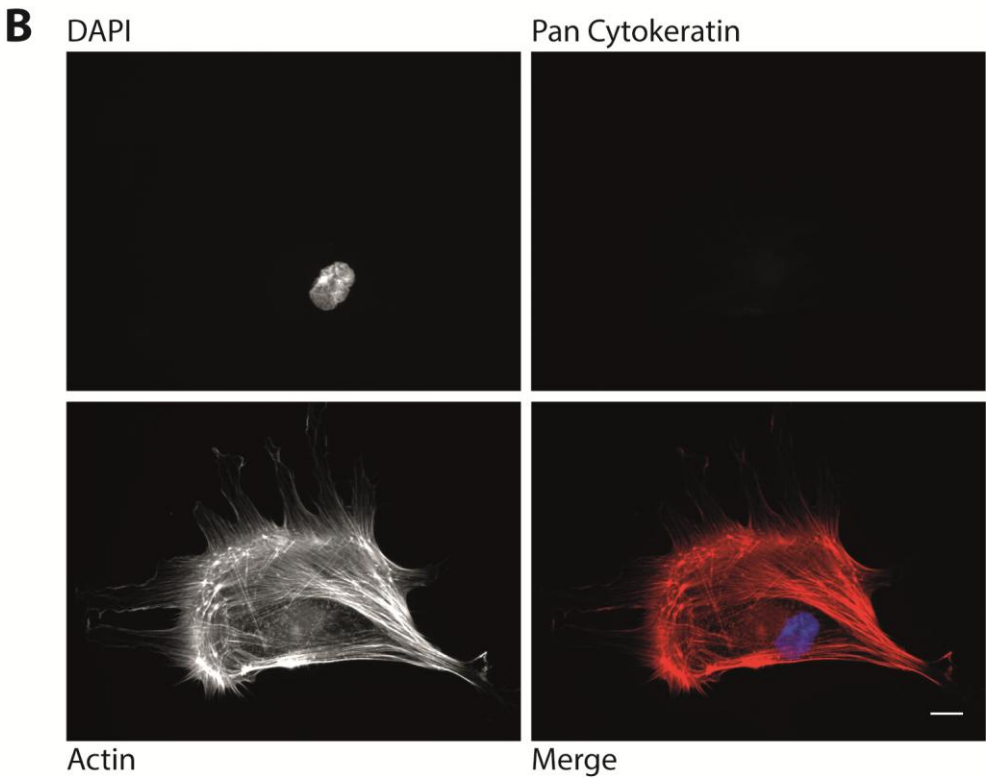
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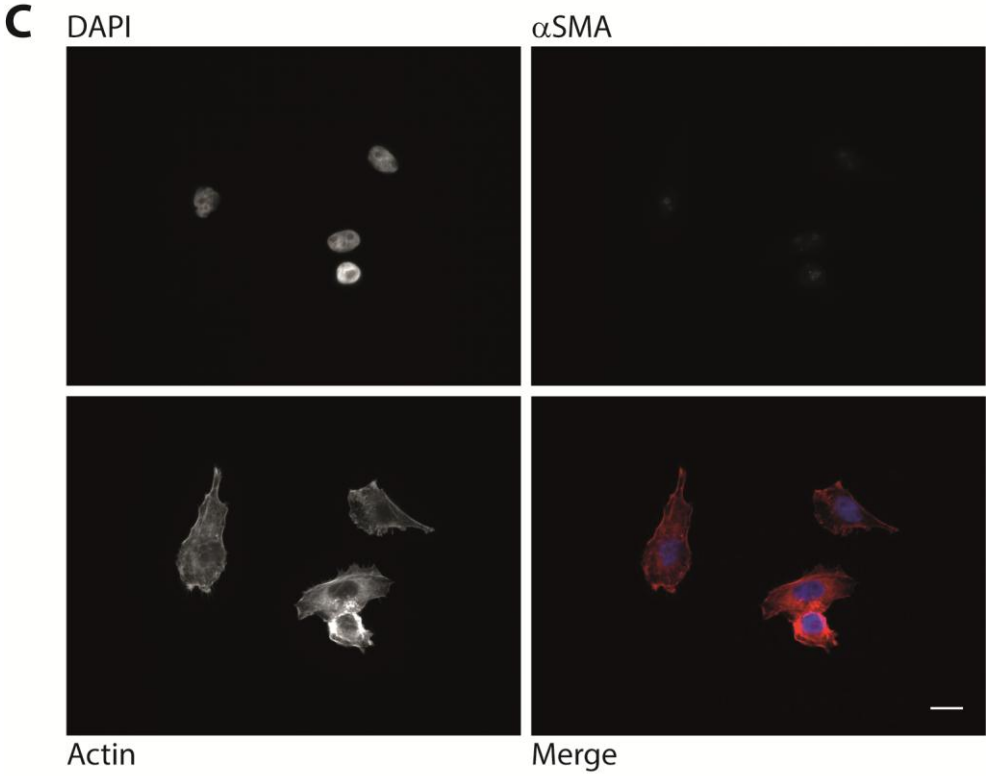
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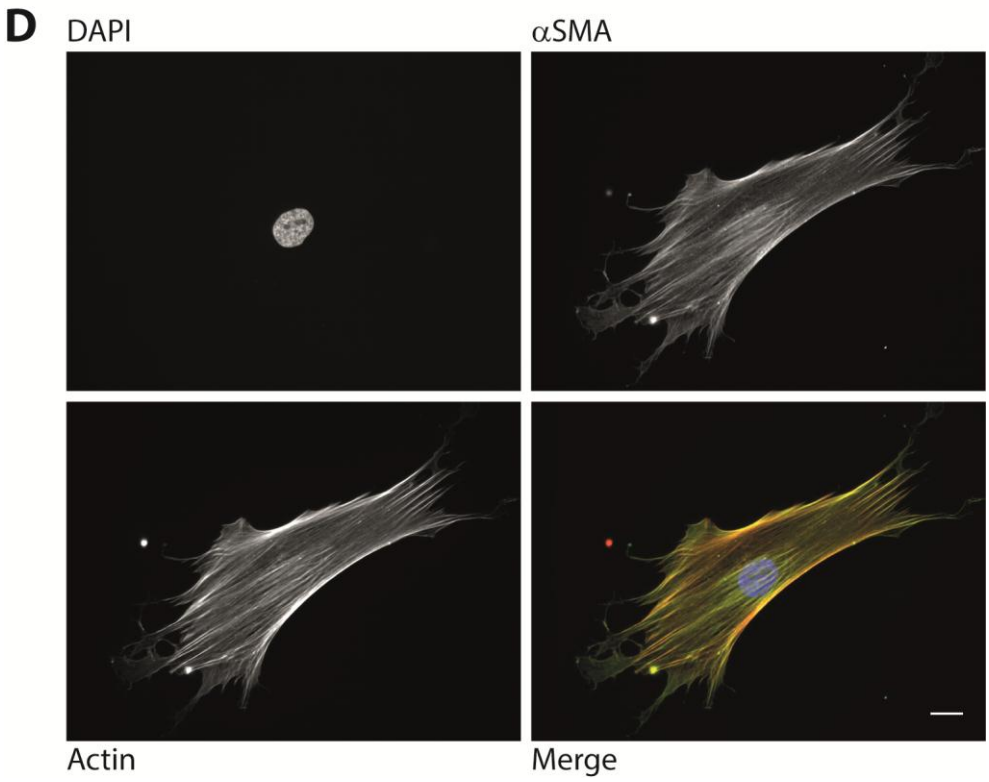
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Figure 6.1: Characterisation of PS-1 and PaTu8988T cells. Both PS-1 and PaTu8988T would be used within the 3D organotypic model. To enable the two cell lines to be distinguished from each other they were subjected to immunofluorescent staining of known stellate/cancer cell markers to ensure the antibodies would be appropriate for future use. Both cell lines were first subjected to immunofluorescent staining for WSS pan-cytokeratin. PaTu8988T (A) but not PS-1 (B) had observed expression of this protein. Conversely, when stained for α-smooth muscle actin, PaTu8988T (C) lacked expression and PS-1 (D) could be seen to have global expression of this marker of stellate cell activation. Scale bar = 10μm.

cells, which had a high level of expression (**figure 6.1D**). These results confirm that these antibodies were suitable for staining the organotypic models with in order for clear identification of each separate cell line.

6.2.2 Optimisation of the organotypic model

3D organotypic models are easily manipulated to better replicate conditions found *in vivo* and can be altered depending on the specific parameter under investigation. These organotypic culture systems involve the generation of a synthetic stroma, which are composed primarily of collagen and Matrigel™ embedded with fibroblasts or stellate cells. Collagen is known to be one of the main components of the ECM and desmoplastic tissue contributing to pancreatic cancer progression (Shields et al., 2011) and Matrigel™ has been shown to enhance cancer cell invasion in 3D (Nystrom et al., 2005) by increasing the diversity of the organotypic matrix (Nurmenniemi et al., 2009).

Two widely used models are the submerged organotypic model and the air-liquid model. Within this study the air-liquid model was used, where the 'gel' is raised onto a grid (**figure 6.2**), which enables the specific study of invasion into the matrix. This model has been widely used to study both SCC (Gaggioli et al., 2007; Nystrom et al., 2005; Zanivan et al., 2013) and pancreatic cancer (Coleman et al., 2014; Froeling et al., 2010; Froeling et al., 2009). There were a number of parameters within the set up of the organotypic, which were tested in order to optimise the procedure. These included the brand of collagen used, the number and placement of stellate cells and the length of time before raising the gel. The altered parameters are

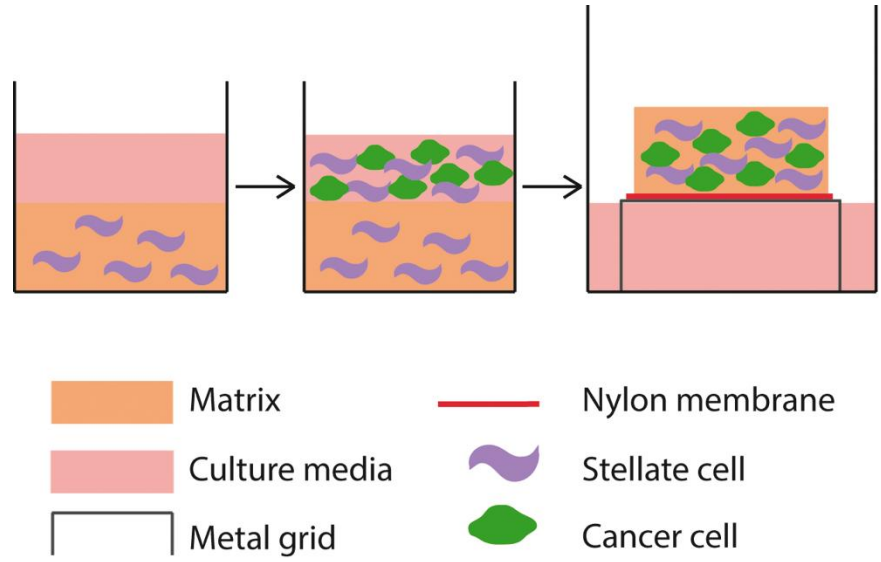


Figure 6.2: Raised air-liquid organotypic model. The raised air-liquid organotypic model was used to study pancreatic cancer cell invasion within this study. Whereby, a collage/matrigel matrix was produced with pancreatic stellate cells also being a main constituent. A 2:1 ratio of cancer:stellate cells was subsequently seeded atop the matrix. This was then raised onto a collagen membrane covered grid and fed with culture media from below to encourage invasion down into the matrix. The media was changed every 2 days and after 14 days each gel was harvested and sent for further processing.

outlined below in **table 6.1** where the optimal conditions used for future experiments are marked; chosen as they were found to produce the most stable gel and optimal conditions for cancer cell invasion.

Variable	Information	
Collagen	BD Biosciences Rat tail type I collagen	
	Concentrated collagen	
	Millipore rat tail type I collagen	✓
Stellate cells	2:1 cancer:stellate cell ratio with all stellate cells seeded on top of gel	
	Equal number of cancer:stellate cells with half in gel and half on top with cancer cells	✓
Raising procedure	Cells seeded on top and gel raised 24 hours later	
	Cells seeded on top and gel raised 48 hours later	✓

Table 6.1: Variables tested for optimizing organotypic model

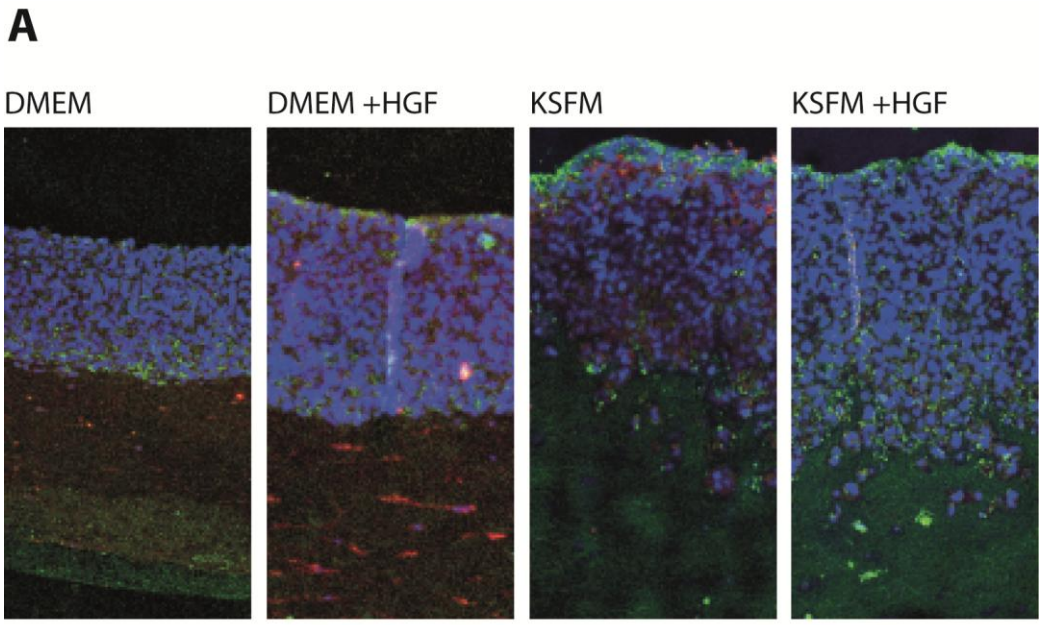
6.2.3 HGF increases invasion of pancreatic cancer cells in 3D organotypic model

Throughout the course of this study, the impact of HGF on PaTu8988T migration and signaling has been investigated. It is well documented that levels of circulating HGF are increased in pancreatic cancer (Ebert et al., 1994) and that is acts as a chemoattractant, enhancing cell migration and invasion (Giacobini et al., 2007; Stella and Comoglio, 1999). It was therefore decided to investigate the effect of using HGF within the organotypic model in order to mimic the elevated levels of HGF that are observed in pancreatic

cancer. With the air-liquid organotypic model, the gel is raised onto a grid and fed from below to encourage invasion. Therefore, HGF was added into the culture media and compared with gels where no HGF was included. It was also decided to compare DMEM with 10% FBS and KSFM supplemented with EGF and BPE, both +/- HGF, in order to find the optimal invasive conditions. It has been shown that the addition of both EGF and BPE increased keratinocyte motility (Fang et al., 1998) and therefore whether the use of this media increased invasion was investigated.

Of the four conditions tested, it was noted that there was a progressive increase in PaTu8988T cells from DMEM minus HGF to KSFM with the addition of HGF. This was calculated through measuring the depth of invasion at three randomly selected areas (imaged by an independent observer) from two independent experiments (**figure 6.3A** and **figure 6.3B**). The areas of invading cells were calculated after exclusion of non-uniform invasion at the edges of the gel. Any areas where obvious artifactual invasion had occurred in identifiable areas (that could be due to processing) were also omitted (see also **section 2.5.1** and **figure 2.3** for details on the analysis procedure).

From these results it demonstrated that HGF increased the invasion of PaTu8988T cells in an organotypic model. It was therefore decided to use these found optimal conditions in future assays where the effect of PAK4 knockdown on cancer cell invasion would be studied.


A
DMEM
DMEM +HGF
KSFM
KSFM +HGF

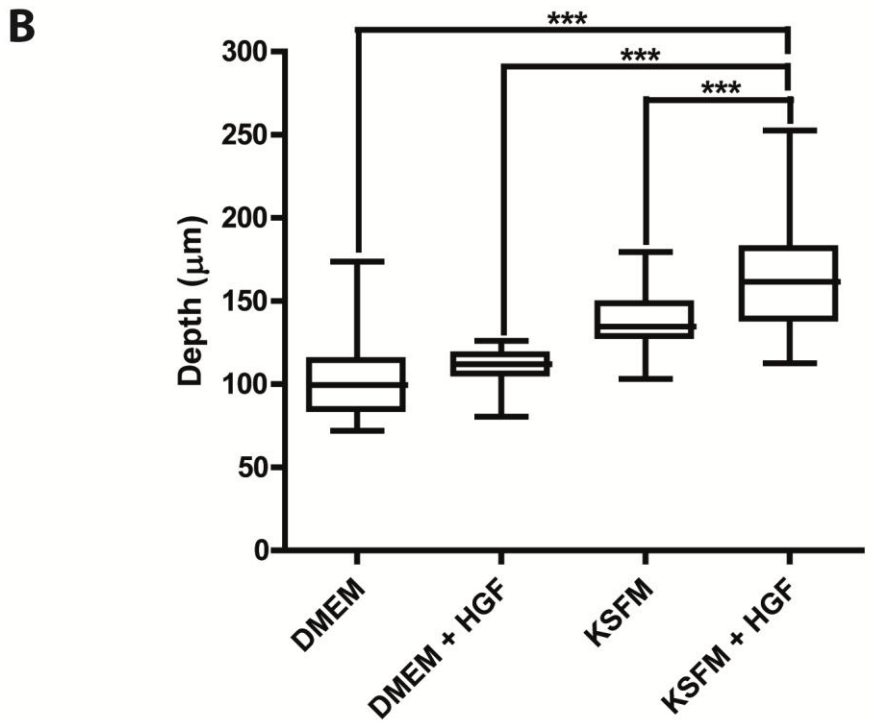

B
***
***
***
300
250
200
150
100
50
0
Depth (μm)
DMEM
DMEM + HGF
KSFM
KSFM + HGF

Figure 6.3: Addition of HGF promotes invasion of pancreatic cancer cells in 3D. Four conditions were tested in order to investigate the optimal conditions for pancreatic cancer cell invasion in the 3D organotypic model. These involved using DMEM and KSFM both +/- HGF to feed the model from below once raised onto the grid. Media was changed every two days. After processing, gels were stained and the depth of invasion measured at random points along the images from two independent experiments. An increase in invasive potential was observed from DMEM -HGF to KSFM +HGF. Therefore, it was decided to use this media to feed all subsequent gels in future assays. Statistical significance was calculated using Student's *t*-test where *** $P < 0.0001$

6.2.4 siRNA mediated depletion of PAK4 has no significant impact on PaTu8988T cellular proliferation

It has been well documented that PAK4 plays a significant role in embryonic development; PAK4 knockout mice are embryonic lethal and die by embryonic day 11.5 (Qu et al., 2003). Conditional knockout of PAK4 in the central nervous system of mice also showed that PAK4 was required for proliferation of neural progenitor cells (Tian et al., 2011b) and it has also been shown that PAK4 functions are required for normal development of the mammalian heart (Nekrasova and Minden, 2012).

Regarding cancer, it has been shown that a reduction in PAK4 in MDA-MB-231 cells resulted in decreased proliferation, which was determined by a cell growth assay (Wong et al., 2013); a result that was amplified in low serum conditions. Overexpression of PAK4 in choriocarcinoma cells increased proliferation significantly and siRNA mediated knockdown conversely saw a reduction (Zhang et al., 2011a). It has also been shown that siRNA mediated depletion of PAK4 in Hep-2 laryngeal carcinoma cells (Sun et al., 2013) and pharmacological inhibition of PAK4 by LCH-7749944 in gastric cancer cells (Zhang et al., 2012) both resulted in a decreased proliferative rate. PAK4 regulation of proliferation has also been observed in ovarian cancer (Siu et al., 2010a) and colon cancer cell lines (Tabusa et al., 2013). Prior to undertaking the investigation of PAK4 depletion on PaTu8988T invasion in the 3D organotypic model, whether it had an effect on proliferation in this cell line was first studied.

More recently is has been shown that PAK4 promotes proliferation of pancreatic cancer cells via AKT and ERK activation of the NF-κB pathway (Tyagi et al., 2014). Therefore, to study the impact of PAK4 knockdown on PaTu8988T proliferation, cells were subjected to siRNA-mediated depletion of PAK4 and subsequently replated onto collagen coated tissue culture plates. Cells were incubated for 72 hours and an MTT assay conducted which detected the number of viable cells. Contrary to previous results, there was no significant difference in cellular proliferation observed in PAK4 knockdown cells when these were compared to both control and WT cells (**figure 6.4**). Although these results were different from those observed in other published studies, within our laboratory it has also been observed that stable PAK4 knockdown breast cancer cells had little observed difference in proliferation. These observed results would mean that if PAK4 knockdown has no impact on PaTu8988T cell proliferation, that any observed differences in invasion could not be due to a decreased proliferative rate.

6.2.5 PAK4 knockdown reduces invasion of PaTu8988T cells in 3D

PAK4 is known to regulate the cytoskeleton in response to its interaction with Cdc42 (Abo et al., 1998). However, PAK4 is also known to interact with GEF-H1 (Callow et al., 2005), LIMK (Dan et al., 2001) and Gab1 (Paliouras et al., 2009). These interactions are, in part, responsible for PAK4 driven cell migration and invasion. PAK4 has been shown to be involved in endothelial cell invasion in 3D collagen matrices, downstream of Cdc42 (Koh et al., 2008) and siRNA suppression of PAK4 inhibited HT1080 cell invasion in 3D

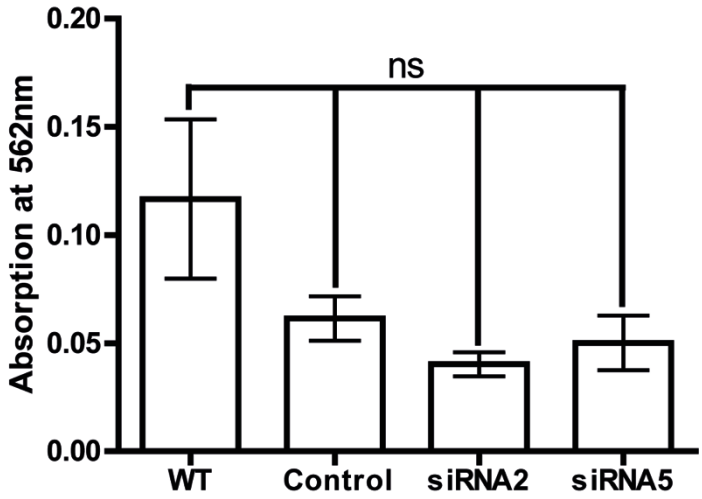
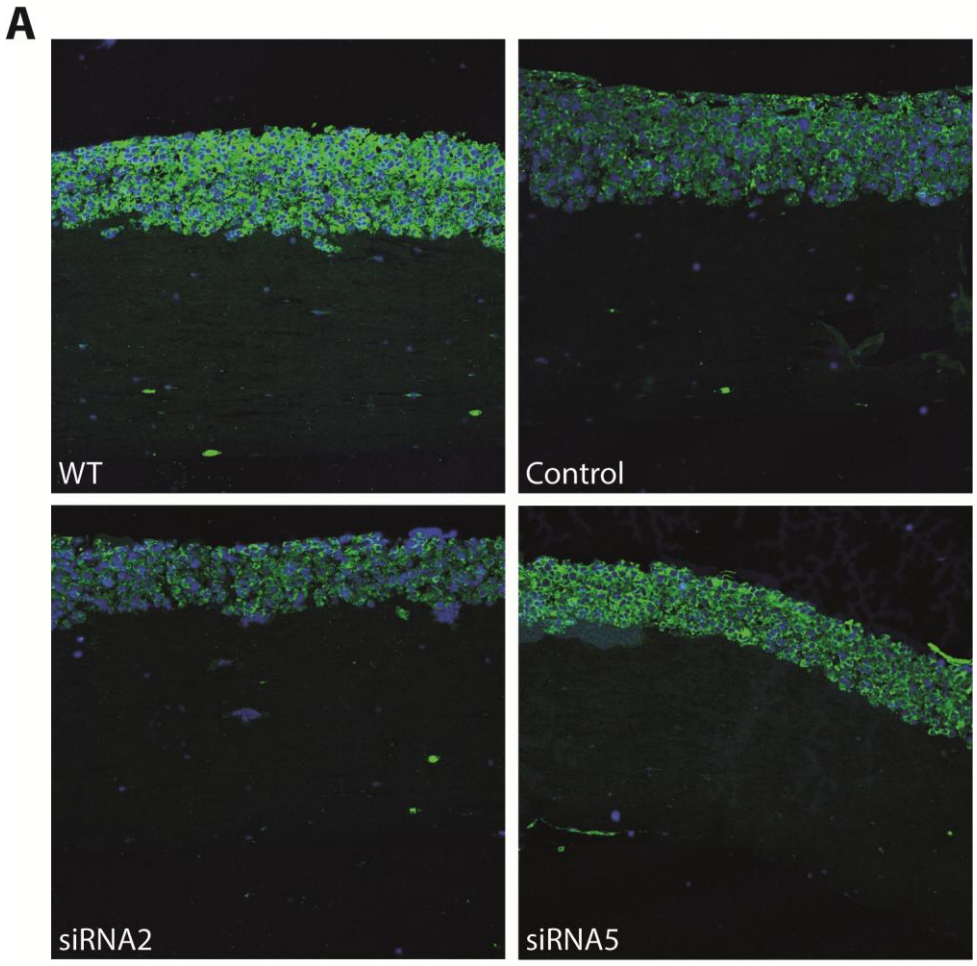
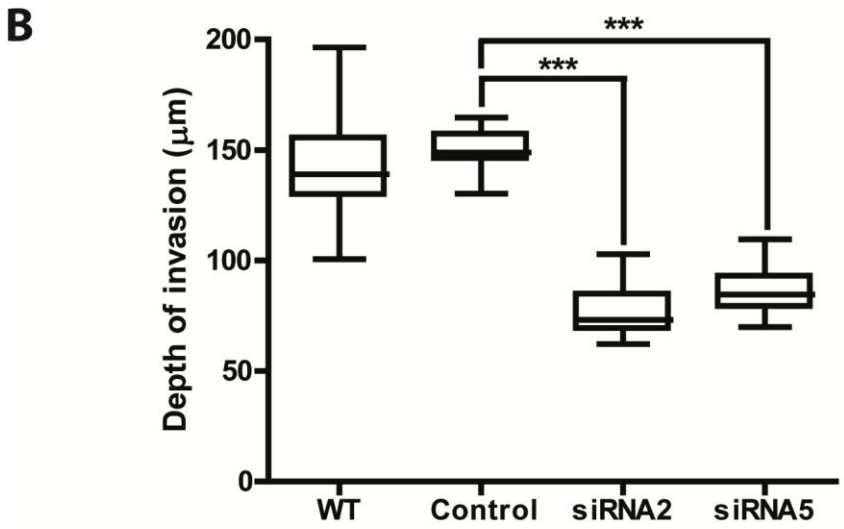


Figure 6.4: Depletion of PAK4 has no significant impact on PaTu8988T cellular proliferation. After siRNA-mediated depletion of PAK4, PaTu8988T cells were subjected to an MTT assay to look at the effect of PAK4 knockdown on cellular proliferation. PAK4 knockdown cells were compared with both WT and control siRNA cells after 72 hours. There was no significant difference observed after results obtained over three independent experiments were quantified.

collagen matrices (Fisher et al., 2009). There are also many studies that strongly implicate abberant PAK4 signalling is involved in cancer cell invasion, including in endometrial (Lu et al., 2013b), ovarian (Siu et al., 2010a), glioma (Kesanakurti et al., 2012), gastric (Guo et al., 2014b) and breast (Wong et al., 2013) cancers . In addtion, a recent study showed that PAK4-depleted A431 cells had significantly decreased invasion in an organotypic model (Zanivan et al., 2013).

To investigate the role of PAK4 on pancreatic cancer cell invasion, PaTu8988T cells were first subjected to siRNA-mediated depletion of PAK4 before being used as previously described in the organotypic model. Three gels from three independent experiments were analysed to compare PAK4 knockdown cell invasion with WT and control siRNA treated cells. As before, the depth of invasion was measured at three randomly selected areas from each experiment, after exclusion of non-uniform or obvious artifactual invasion areas of the gel. This thickness of the cell layer is observably less after PAK4 knockdown in comparison to both WT and control cells (**figure 6.5A**). In addition, the depth of invasion was calculated and demonstrated that there was a significant reduction in PaTu8988T cell invasion into the matrix after depletion of PAK4 (**figure 6.5B**). This was apparent with both siRNA oligonucleotides used in comparison to control siRNA-treated cells. Looking at the interface between the invading cancer cells and the matrix, it was also shown that there was an increasingly disorganised architecture at the invasive edge in control cell compared to PAK4 knockdown cells (**figure**


A
WT
Control
siRNA2
siRNA5


B
Depth of invasion (µm)
200
150
100
50
0
***
***
WT
Control
siRNA2
siRNA5

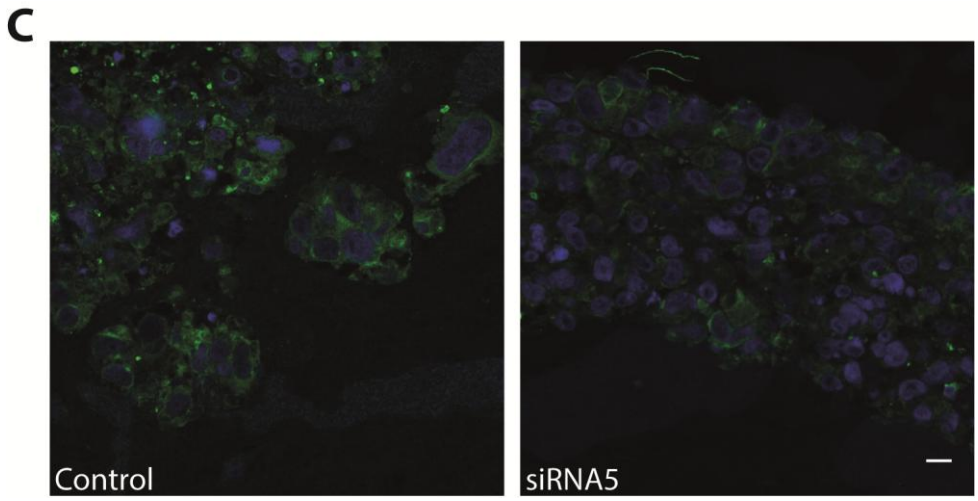


Figure 6.5: PAK4 depletion leads to a reduction in PaTu8988T cell invasion in 3D. PaTu8988T cells were used in the organotypic model after depletion of PAK4. After 14 days the gels were harvested and sent for processing. Immunofluorescent staining was used to look specifically at cancer cell invasion. Images show staining for DAPI (blue) and WSS pan-cytokeratin (green) which is specific to cancer cells (A). Results from three independent experiments were quantified after measurements at randomly selected points were measured (B). Results demonstrate a significant reduction in PaTu8988T cell invasion after siRNA-mediated knockdown of PAK4 in comparison to control siRNA-treated cells. (C) Magnification using a 40X lens imaging the interface between the cell:matrix border shows that there is a more disorganised cell architecture in control cells in comparison to PAK4 knockdown cells, with cell clusters breaking away from the main cell group to invade down into the matrix. Statistical significance was calculated using Student's *t*-test where *** $P < 0.0001$

6.5C), with more cells evidently breaking away and invading as small groups of cells down into the matrix although the same pattern on invasion observed in preliminary experiments (**figure 6.3**) was not observed.

It is known that resistance to apoptosis is a key process in oncogenesis and it has been shown that PAK4 promotes cell survival via several mechanisms (King et al., 2014). It has been shown that cells overexpressing PAK4 are able to evade apoptosis (Gnesutta et al., 2001), which can be kinase independent through antagonism of caspase 8 activation or kinase dependent phosphorylation of the proapoptotic protein Bad (Gnesutta and Minden, 2003; Gnesutta et al., 2001). In contrast, PAK4 is also known to stimulate the activation of pro-survival pathways induced by TNF-α (Li and Minden, 2005).

Knowing that the antiapoptotic functions of PAK4 play a key role in tumorigenesis, the organotypic gels were stained for cleaved caspase 3, which is a marker of apoptosis. This was to determine if there was any visible differences in the number of apoptotic cells present before/after PAK4 knockdown and whether cell death contributed to the differences in invasion observed. The gels for WT, control and both PAK4 knockdown conditions were stained with both cleaved caspase 3 and DAPI and the images compared (**figure 6.6**). There was no marked difference in the expression of cleaved caspase 3 observed across all four conditions, although staining did appear to be weaker in siRNA2 treated cells. However, of note was the increased expression that appeared at the top of each gel. Due to the set up

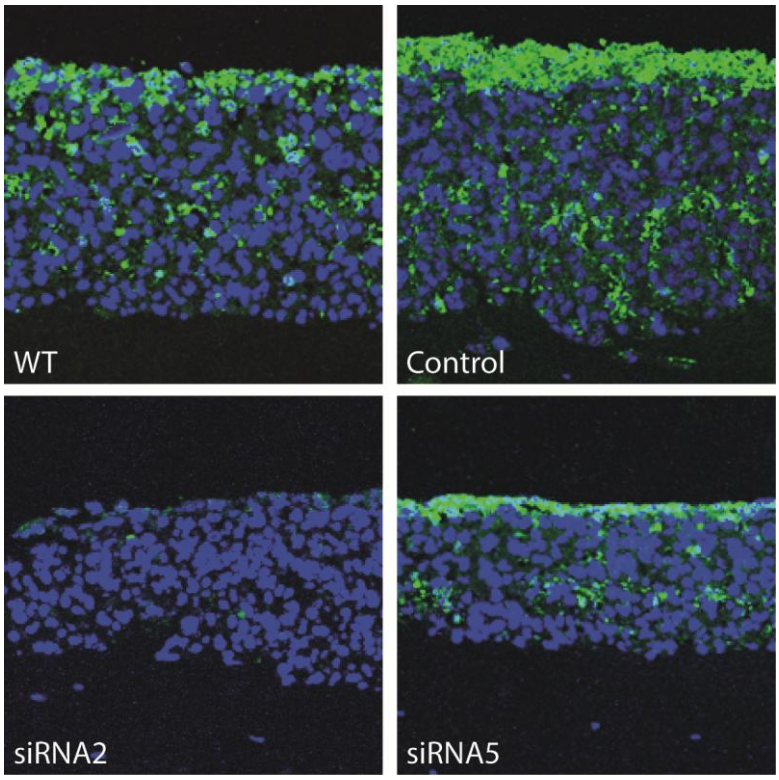


Figure 6.6: Cleaved caspase 3 staining of 3D organotypic model. To investigate whether apoptotic cell death contributed to the observed differences in invasion observed after PAK4 knockdown, sections were stained for DAPI (blue) and cleaved caspase 3 (green). Of note, was the strong staining at the top of the sections. There appeared not to be any other observed differences in the number of apoptotic cells throughout the section. However, there appeared to be only very few cleaved caspase 3 positive cells in siRNA2 treated cells.

of the experiment, the exposure of the top of the gel could account for the pattern of apoptotic cells observed.

Having investigated the effect of PAK4 knockdown on proliferation via an MTT assay previously. The organotypic sections were also stained for Ki67, which is a marker of actively proliferating cells. This was used as another way of examining whether there could be an observed difference in cellular proliferation once cells were used within the organotypic and at a longer time frame. There appeared to be only limited observed differences in the expression patterns of Ki67 across the four conditions tested (**figure 6.7A**), but after quantification using ImageJ there appeared to be a slight decrease in ki67 positive cells after PAK4 knockdown especially siRNA5 (**figure 6.7B**).

6.2.6 Inhibition of PI3K phenocopies PAK4 knockdown invasion patterns

Throughout this investigation, the links between PAK4 and the PI3K pathway have been studied. This has, in part, included the comparison between PAK4 knockdown and pharmacological inhibition of PI3K via treatment with LY294002. Having investigated the effect of PAK4 knockdown in a 3D organotypic model, the effect of PI3K inhibition was next studied.

As before, cellular proliferation was first investigated. It is known that the PI3K pathway plays a key role in cellular proliferation (Hemmings and Restuccia, 2012), therefore it was not unexpected for there to be a significant reduction in cellular proliferation as observed with the results from the MTT

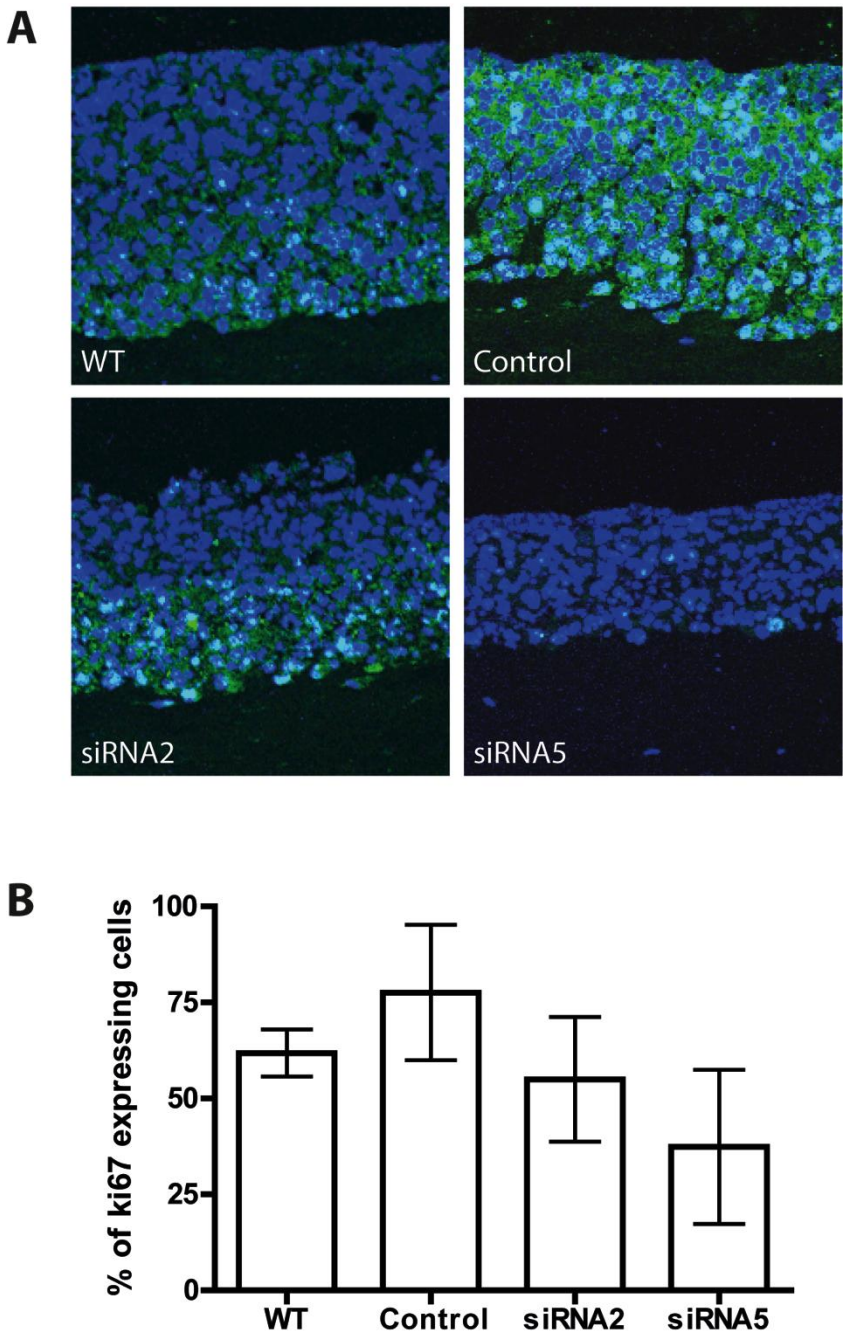


Figure 6.7: Ki67 staining of 3D organotypic model. In addition to the MTT assay, Ki67 staining was performed on sections to look at actively proliferating cells (**A)**. Sections were co-stained with DAPI (blue) and Ki67 (green). WT, control and siRNA2 treated cells appeared to have actively proliferating cells throughout the section. Qualitative analysis revealed that there were however, only a small number of observed ki67 positive cells in siRNA5 treated PaTu8988T cells. N=1 but two images from each condition were analysed and quantified using ImageJ (**B**) which confirms a slight decrease in the percentage of ki67 expressing cells/actively proliferating cells after PAK4 knockdown, particularly with siRNA5.

assay (**figure 6.8A**). The PI3K inhibitor has been widely used in the study of cellular proliferation, in mouse embryonic stem cells treatment lead to a marked decrease in cellular proliferation and cell cycle arrest, with an accumulation of cells in the G_1 stage of the cell cycle (Lianguzova et al., 2007). Pharmacological studies have also shown a significant reduction in cellular proliferation in bladder (Wu et al., 2011a), nasopharyngeal carcinoma (Jiang et al., 2010), ovarian (Hu et al., 2000) and also colon and pancreatic cancer cells (Semba et al., 2002) under nutrient starvation conditions (Izuishi et al., 2000). Therefore, the significant decrease in PaTu8988T cellular proliferation, that was observed, is what would be expected.

Knowing that LY294002 can affect both cellular proliferation and induce apoptosis, these factors would be considered upon analysing results obtained from the organotypic data set. As before, three independent experiments were conducted using the PI3K inhibitor, where LY204002 was added to the media feeding the gel from below. As a control, DMSO was used as an additive at the same concentration. The same analysis parameters were used with regards to the measurements taken to calculate the invasive cell depth.

The results were gathered and a significant reduction in PaTu8988T cell invasion was observed after cells had been subjected to pharmacological inhibition of PI3K in comparison to the DMSO control cells (**figure 6.8B**). Only a very thin layer of cells was apparent in images taken of the PI3K inhibitor treated cells in comparison to the DMSO control cells (**figure 6.8C**).

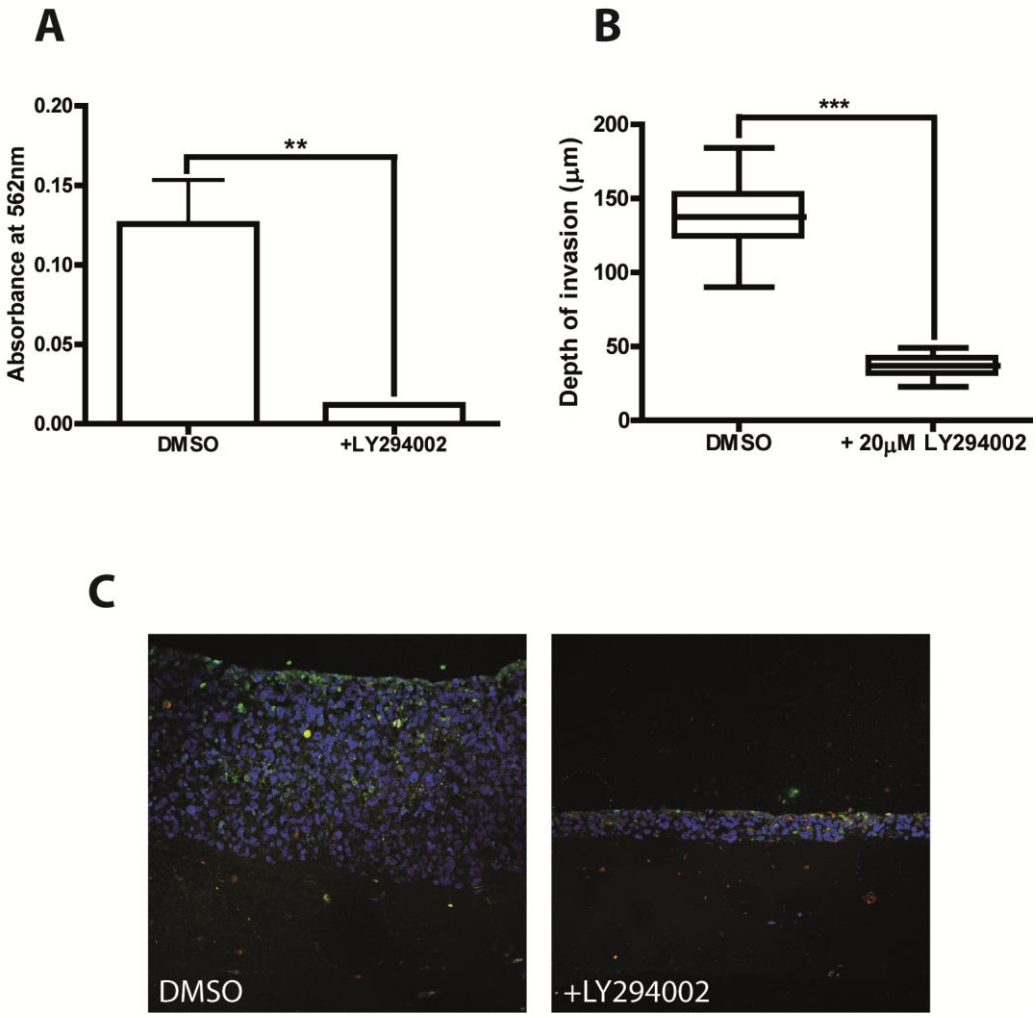


Figure 6.8: Pharmacological inhibition of PI3K phenocopies invasion patterns of PAK4 knockdown cells. The PI3K inhibitor, LY294002, was used to study the impact of pharmacological inhibition of PI3K on PaTu8988T cell invasion. (A) An MTT assay was performed and demonstrated that there was a significant reduction in cellular proliferation after treatment with LY294002 in comparison to DMSO control cells. (B) Sections of a 3D organotypic model were analysed and quantitative analysis revealed that there was a significant reduction in PaTu8988T cell invasion after PI3K inhibition. (C) Sections stained for DAPI (blue), WSS pan-cytokeratin (green) and α-SMA (red) revealed a reduced invasion into the matrix. Results and images shown are data gathered from three independent experiments. Statistical significance was calculated using Student's *t*-test where ** $P < 0.002$ and *** $P < 0.0001$

6.3 Discussion

Within this chapter, a 3D organotypic model of pancreatic cancer was used to study the effect of PAK4 knockdown on PaTu8988T cell invasion. The model provides a platform in which to study cellular behavior in an environment that better replicates conditions found *in vivo*. This therefore provides a more complete insight into the effects of the chosen variable using an assay that is reproducible. The air-liquid organotypic model is a widely used 3D model in which to study cancer cell behavior. It has been used in numerous studies to investigate cancer cell invasion including pancreatic cancer (Coleman et al., 2014; Froeling et al., 2010; Froeling et al., 2009). Although, the model has been well characterised, further optimisation allowed for the production of more stable gel matrix, which therefore made the procedure more consistent and allowed for results to have increased reproducibility.

To enhance invasion, initial studies of PaTu8988T cell invasion involved using WT cells and altering the culture media used to feed the organotypic gel from below. In previous chapters, it has been demonstrated that PaTu8988T cells responded to HGF with an increased velocity in random 2D migration assay. It was therefore decided to transfer these 2D findings into the 3D model, whereby media supplemented with HGF at the same concentration was compared to media where no HGF was added. It was also decided to test another culture media, KSFM. KSFM has additional growth factors used to supplement the media, including EGF, and KSFM +/- HGF

was used in addition to the DMEM +/- HGF in order to find optimal conditions for promoting invasion of the PaTu8988T cells in the organotypic model.

Across the four conditions, a progressive increase in invasion was observed from DMEM -HGF to DMEM +HGF, which increased further in KSFM -HGF with the most invasion being observed when KSFM supplemented with HGF was used to feed the gel from below. The additional growth factors contained within the media therefore must promote invasion. From previous studies and work throughout the course of this investigation, it has been observed that HGF increases pancreatic cell migration (Ebert et al., 1994; Matsushita et al., 2007; Ohba et al., 1999). Therefore, it was expected to have a similar result when transferred to the 3D model. Although it has not been investigated within this study, as well as the HGF receptor C-met being upregulated, EGFR is known to be overexpressed in pancreatic cancer (Oliveira-Cunha et al., 2011). Numerous studies have also shown that an increase in pancreatic cancer cell migration occurs in response to EGF (Liu et al., 2012; Ouyang et al., 2013; Stock et al., 2014). Therefore, the results observed here, whereby there was an increase in PaTu8988T cell invasion when KSFM supplemented with both EGF and the additional HGF, are what would be expected.

Prior to using PAK4 depleted PaTu8988T cells in the organotypic model, an MTT assay was carried out to investigate the impact of PAK4 knockdown on PaTu8988T proliferation. This could therefore be used to determine whether any potential differences observed in invasion were due to a decreased

invasive potential or due to a decreased proliferative capacity. It has been shown in numerous studies that PAK4 impacts on cell cycle progression through regulation of the cell-cycle regulatory protein, p21, which plays a significant role in controlling the G_1 to G_2/M transition (Nekrasova and Minden, 2011) and also through regulation of spindle positioning throughout mitosis (Bompard et al., 2013). As previously stated, it is also known that PAK4 depletion or inhibition in various cancer cells leads to a decrease in cellular proliferation. This has been studied in breast (Wong et al., 2013), gastric (Zhang et al., 2012), ovarian (Siu et al., 2010a) and colon (Tabusa et al., 2013) cancer cell lines, among others. Therefore, it was somewhat surprising to find that siRNA-mediated depletion of PAK4 in the PaTu8988T cells had no significant impact on cellular proliferation/viability after quantifying results obtained from a 72-hour cell growth assay.

As the organotypic assays were to be carried out over a period of 14 days, it could be argued that 72 hours may not provide a long enough time frame in which to study changes in proliferation accurately. However, as demonstrated in previous results chapters, knockdown of PAK4 using both of the siRNA oligonucleotides is evident after only 24 hours. Therefore, any impact PAK4 depletion had on cellular proliferation would most likely be evident by 72 hours. In addition, unpublished data using stable PAK4 knockdown breast cancer cell lines has also shown little change in cellular proliferation when conducting the same cell growth assay. It is also noted, that the stable PAK4 knockdown breast cancer cell lines could be maintained in long term cell culture. In a study of colon cancer, it was also demonstrated

that knockdown of either PAK4 or PAK1 impacted on cellular proliferation in a cell line dependent manner. Of the seven cell lines used, six showed a decrease in cellular proliferation after PAK4 knockdown and only two showed a decrease after PAK1 knockdown (Tabusa et al., 2013). A recent study of pancreatic cancer, which demonstrated promotion of proliferation through PAK4 activation of the NF-κB pathway (Tyagi et al., 2014) had not used the PaTu8988T cell line. This could therefore go some way as to explain the results observed here and it could be hypothesised that the effects of PAK4 depletion on cellular proliferation are cell line dependent.

Subsequent to the proliferation studies, the effect of PAK4 knockdown in the 3D model of pancreatic cancer was next investigated. It has been shown that knockdown of PAK4 in A431 cells resulted in decreased invasion (Zanivan et al., 2013) in a similar 3D organotypic model. However, to date, the effect of PAK4 knockdown using pancreatic cancer cell lines in this system has yet to be investigated. The results obtained through these studies showed that there was a significant decrease in PaTu8988T cell invasion down into the matrix, in comparison to WT and control cells. However, the same pattern of invasion as demonstrated in earlier experiments was not reproducible. Minimal invasion of cells, either as single cells, strands or clusters was not observed at a significant level. Despite MTT assays carried out suggest minimal impact of PAK4 knockdown in the PaTu8988T cell line, the results observed within the organotypic model imply an effect on proliferation as a thinner layer of cells is evident in PAK4 knockdown samples, in comparison to both WT and control conditions. This may be due to differences in cell

behaviour in 2D assays to the 3D environment of the organotypic model. Although some increasingly disorganised architecture at the cell/matrix interface was observed, no quantifiable patterns of invasion were observed. These results therefore prevent full conclusions as to the effect of PAK4 knockdown on PaTu8988T cell invasion in 3D environment. Further investigation into the effect of PAK4 knockdown on longer-term proliferative effects as well as invasion would therefore be advisable.

For qualitative analysis, sections from each condition were stained for ki67 (a marker of actively proliferating cells) and cleaved caspase 3 (expressed by apoptotic cells). The four different conditions under examination demonstrated similar expression patterns. Regarding the expression of cleaved caspase 3, cells with positive expression of this protein appeared to be mainly concentrated at the top of the gel. It was hypothesised that due to the experimental set up, where the gel is exposed to the environment, cells that were atop the gel could be under increased stress and this could account for the concentration of cells expressing this marker towards the top of the gel. However, taken together, these novel findings highlight that PAK4 could be heavily implicated in the invasion of pancreatic cancer cells.

In addition to investigating the impact of PAK4 depletion on PaTu8988T cell invasion and complimenting previous work, the effect of pharmacological inhibition of PI3K by LY294002 was investigated. As with PAK4 knockdown cells, an MTT assay was carried out in order to look at changes in cellular proliferation/viability. It is known that the use of LY294002 leads to a

significant reduction in cellular proliferation and induces programmed cell death in various cancer cell lines, including pancreatic (Izuishi et al., 2000; Semba et al., 2002). Therefore, the significant reduction in LY294002 treated PaTu8988T cells, compared to the DMSO control cells was as predicted as it is known that both cellular proliferation and cell cycle progression are PI3K dependent in pancreatic cancer and also that this signaling cascade has antiapoptotic properties (Perugini et al., 2000). Following the proliferation studies, the effect of pharmacological inhibition of PI3K was investigated in the organotypic model. Media was supplemented with either DMSO, as a control, or LY294002. Following quantification there was a significant reduction in the depth of invasion following treatment with LY294002. Having previously observed the significant reduction in cellular proliferation the difference in invasion is as predicted. In addition, visualization of the stained sections revealed a marked decrease in cell number. Again this can be explained by the reduction in cellular proliferation and hypothesised increase in programmed cell death.

Within this chapter, novel data has shown that there is a requirement for PAK4 within pancreatic cancer cell invasion, as observed through the use of an established 3D organotypic model.

6.4 Future Directions

It was observed within this chapter that the depletion of PAK4 from PaTu8988T cells had no significant impact on cellular proliferation. It was hypothesised that this could be cell line specific as differential responses have been observed in other cancer cell lines. It would therefore be beneficial to study the effect of PAK4 knockdown in a panel of pancreatic cell lines to determine as to whether this hypothesis is correct. However, due to the nature of this study, the fact that the depletion of PAK4 had minimal effects of PaTu8988T proliferation did infer that alterations in invasive capacity was due to the reduction in PAK4 protein levels. However, from qualitative analysis of the organotypic images, an effect of proliferation could be suggested, contrary to the MTT assay results. This could be due to differences in cell behaviour in 3D environments in comparison to 2D. It would therefore be recommended to conduct proliferation assays with cells embedded in a collagen matrix to provide a similar environment to the organotypic model in which to study the effect of PAK4 knockdown on PaTu8988T cell proliferation. In addition, it may be advised to study other markers of proliferation/cell cycle. Previous studies that demonstrated a correlation in PAK4 knockdown and decreased proliferation also observed a reduction in cyclin D1 (Siu et al., 2010a; Tabusa et al., 2013; Zhang et al., 2011a), therefore investigating the levels of this protein in the pancreatic cell lines after PAK4 knockdown could be beneficial.

The 3D organotypic model of pancreatic cancer used within this study is an appropriate platform in which to study cancer cell invasion and bridges the

gap between 2D *in vitro* cell-based assays and complex *in vivo* models. It would of course be ideal to be able to use *in vivo* mouse models to investigate the role of PAK4 in pancreatic cancer progression as they provide an environment in which to study the disease in all its stages in an environment that is more comparable to that found in humans. However, the long latency periods involved make these sorts of studies costly and they are less easily manipulated than the organotypic models. Further use of these organotypic models could therefore be advantageous to study pharmacological inhibition of PAK4 for example.

Staining of organotypic sections for cleaved caspase 3 was carried out for qualitative analysis of apoptotic cells. It would be useful to carry out more quantitative analysis of apoptosis. Various caspase assays are commercially available which could be used to investigate the effect of PAK4 knockdown on programmed cell death within the cell line used to provide further insight.

It is known that cross-talk between stellate cells and cancer cells contributes to pancreatic cancer disease progression. It would therefore be of interest to investigate whether PAK4 contributes to this too. A coculture model to investigate hepatocytes and hepatic stellate cells has been used to demonstrate bi-directional crosstalk, resulting in gene deregulation and changes to the microenvironment through induction of VEGFA and MMP-9 (Coulouarn et al., 2012). In addition, studies utilizing conditioned media from pancreatic cancer cells have been shown to stimulate pancreatic stellate cells and increased production of MMP-2 (Bachem et al., 2005;

Schneiderhan et al., 2007). An interaction between PAK4 and MMP-2 has been previously reported to be involved in the invasion of glioma (Kesanakurti et al., 2012), Therefore it could be hypothesised that this could also play a role in pancreatic cancer progression and may be worth further investigation. However, the results obtained throughout this results chapter have provided novel insight into the role of PAK4 on pancreatic cancer cell invasion and have validated the need for further investigation.

Chapter 7
Concluding Remarks

Chapter 7: Concluding remarks

In this study it has been demonstrated that PAK4 is overexpressed in a range of pancreatic cancer cell lines, which correlates with an increased expression of c-Met, K-ras and the p85α subunit of PI3K. Furthermore, using the highly invasive cell line, PaTu8988T, it has been shown that these cells respond to HGF with both an increase in the phosphorylation of downstream effector proteins such as Akt and also an increased migrational response. In addition, a novel interaction between PAK4 and components of the Ras/PI3K pathway has been established. Of particular interest was the interaction between PAK4 and p85α, which has been show to be dependent on the proline rich region of PAK4 and how Gab1 may also act as a scaffold between the two proteins. In addition siRNA-mediated depletion of PAK4 was shown to reduce both 2D migration and 3D invasion, which may be through regulation of the downstream effector protein, Akt; these data have also been shown to be phenocopied through pharmacological inhibition of PI3K. Thus, PAK4 may be involved in the PI3K pathway to promote pancreatic cell invasion.

The question posed was how PAK4 was involved within pancreatic cancer progression, with particular interest on how it interacts with the PI3K pathway. There is already considerable evidence to suggest a role for PAK4 in metastasis and invasion in other cell types and more recently links between PAK4 and pancreatic cancer have been investigated (Chen et al., 2008; Kimmelman et al., 2008; Mahlamaki et al., 2004). PAK4 is the most widely studied of the group II PAKs and has been shown to contribute

significantly to cancer cell invasion in gastric cancer (Guo et al., 2014b; Zhang et al., 2012), glioma (Kesanakurti et al., 2012), choriocarcinoma (Zhang et al., 2011a), endometrial (Lu et al., 2013b), prostate (Ahmed et al., 2008; Wells et al., 2010), ovarian (Siu et al., 2010a) and breast cancer (Minden, 2012; Wong et al., 2013) among others, both *in vitro* and *in vivo*. PAK4 is able to promote cancer cell invasion through modulation of the actin cytoskeleton and microtubules. It is known that this regulation occurs through interaction with various proteins including Cdc42, which leads to reorganisation of actin in the formation of filopodia (Abo et al., 1998); GEF-H1 mediated interaction with microtubules allows for increased motility (Callow et al., 2005); phosphorylation of LIMK by PAK4 enables inhibition of the actin filament disassembly protein, cofilin (Dan et al., 2001); and association with the scaffold protein Gab1 leads to modulation of cell migration within lamellipodia (Paliouras et al., 2009).

However, how PAK4 may drive pancreatic cancer cell invasion has yet to be fully elucidated. Genomic studies have shown that PAK4 can be activated by oncogenic K-ras, which is mutated in over 90% of pancreatic tumour samples (Chen et al., 2008) and *in vitro* cell-based assays have shown that shRNA-mediated knockdown of PAK4 in a pancreatic cancer cell line reduced cell migration (Kimmelman et al., 2008). However, the mechanisms underlying these findings have not been fully investigated.

Data presented here demonstrate that PAK4 is able to pulldown K-ras; although links between K-ras and PAK4 in pancreatic cancer have been

established (Chen et al., 2008) and the overlap in signalling networks regulated by K-ras and PAK4 investigated (Gnad et al., 2013), this interaction has not previously been described. Moreover, an interaction between the p85α subunit of PI3K and the proline rich region was established. This novel interaction between PAK4 and p85α was found to be dependent on the proline rich region of PAK4 and the SH3 domain of p85α. Whether Gab1 was involved in this interaction was also investigated, as it is known to bind to both the GID of PAK4 (Paliouras et al., 2009) and the SH2 domain of p85α (Rocchi et al., 1998; Yu et al., 2001). Results revealed that Gab1 knockdown lessened that interaction observed between PAK4 and p85α. However, as binding was observed between just the SH3 domain and PxxP region it was hypothesised that binding between PAK4 and p85α could be both direct and indirect, via the scaffold protein Gab1. The mechanism of PAK4 activation has been the subject of intense study. Recently, an N-terminal region (R^{49}PKP) was found to act as an autoinhibitory pseudosubstrate. It was also found that Src SH3 could activate PAK4 and relieve autoinhibition (Ha et al., 2012). The tertiary structure and binding partners of the SH3 domains of both Src and p85α have shown commonalities previously (Koyama et al., 1993; Shen et al., 1999); this therefore provides scope for activation of PAK4 by pseudosubstrate autoinhibition release through binding to a SH3 domain containing proteins, which could potentially include p85α.

Further to the interaction studies with upstream components of the PI3K pathway, how PAK4 was involved with downstream effector proteins was also investigated. It was shown that depletion of PAK4 resulted in a reduction

of Akt phosphorylation. During the course of this investigation, newly published data has corroborated these findings, whereby a reduction in PAK4 in both NIH3T3 and gastric cancer cells lines resulted in a reduction of Akt at Ser473 (Fu et al., 2014; Gnad et al., 2013). In addition, this phenotype was seen to be rescued by expression of constitutively active K-ras (Gnad et al., 2013). This further supports the hypothesis of a RAS/PI3K/PAK4/AKT pathway, but the comprehensive mechanisms behind PAK4/Akt interplay still remains unclear. Whether PAK4 affects phosphorylation of Akt at Thr308, as well as Ser473, has not yet been investigated and if the regulation of Akt by PAK4 is direct or mediated via other mechanisms requires further study.

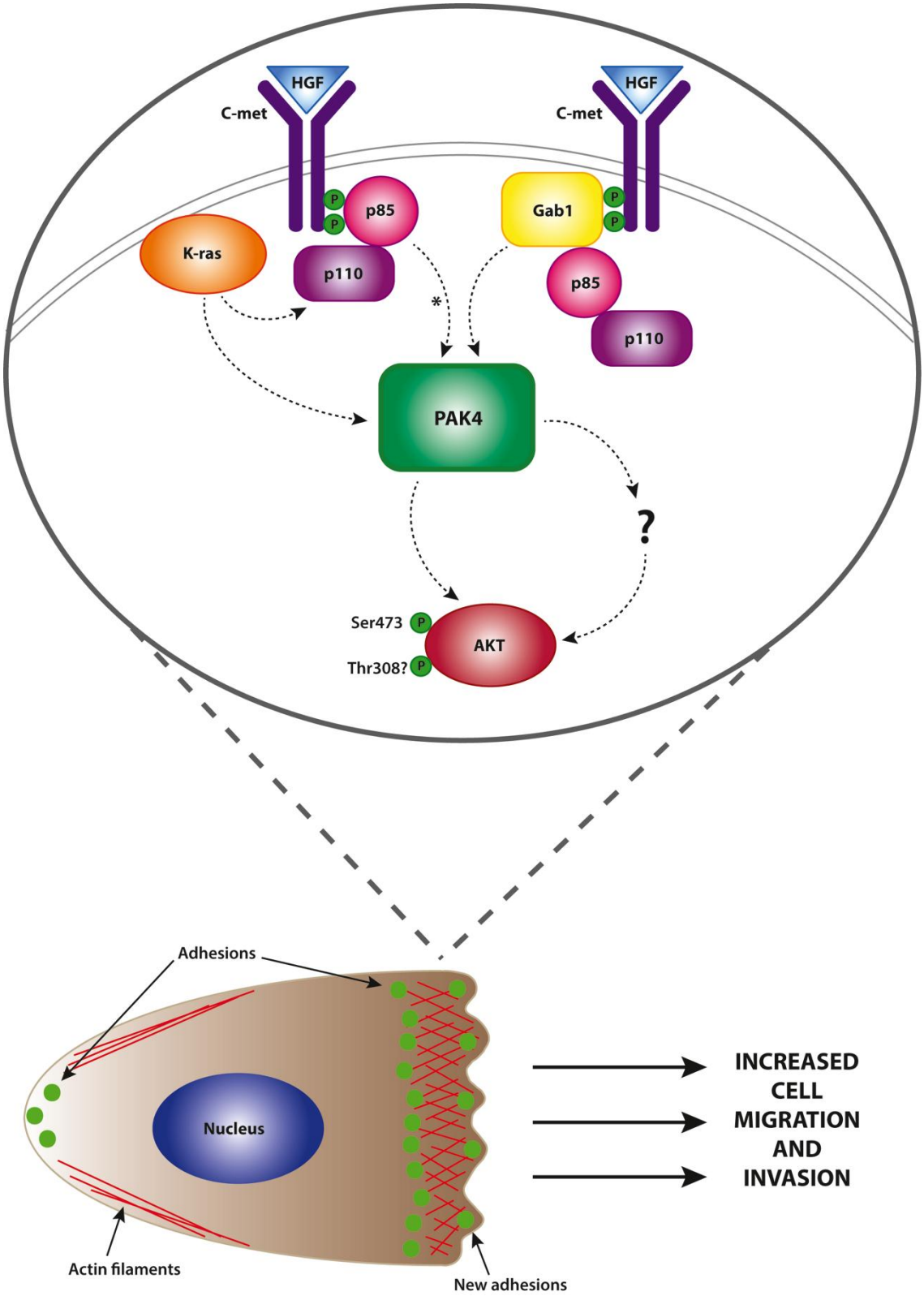
Akt is recruited to the membrane by binding of its PH domain to the PI3K reaction products PIP_2 and PIP_3, and this translocation to the membrane is crucial for Akt activation (Kohn et al., 1996; Sarbassov et al., 2005). It is known that both PDK1 (Higuchi et al., 2008) and the mTORC2 complex (Case et al., 2011; Moore et al., 2011; Sarbassov et al., 2005; Tato et al., 2011) impact on AKT activity through phosphorylation at Thr308 and Ser473, respectively, downstream of PI3K. It has also been shown that PDK1 can interact with PAK1 (King et al., 2000; Riaz et al., 2012; Weber et al., 2004). Indeed, in NIH-3T3 cells, activated PAK1 increased phosphorylation of Akt at both sites, which was shown to be independent of PAK1 kinase activity. It was demonstrated that PAK1 served as a scaffold, binding to both PDK1 and Akt to promote Akt phosphorylation by PDK1. This interaction was increased after growth factor stimulation and was found to promote cell motility (Higuchi et al., 2008). PAK1 has also been shown to affect phosphorylation of Akt at

Ser473. A study using cardiomyocytes revealed a direct interaction between PAK1 and Akt, which resulted in Akt phosphorylation at Ser473 (Mao et al., 2008; Rauch et al., 2011). Despite differences in sequence homology, PAK1 and PAK4 share a number of substrates (Dart and Wells, 2013), so it could therefore be hypothesised that PAK4 affects the phosphorylation of Akt by a similar mechanism. It would therefore be highly beneficial to investigate these possible regulatory mechanisms further.

Furthermore, although typically the PI3K/AKT pathways has been considered primarily to be responsible for survival signalling and proliferation, there is accumulating evidence to suggest that Akt signalling contributes to cellular motility, including in metastatic cancer cells (Higuchi et al., 2001; Kim et al., 2001; Xue and Hemmings, 2013; Xue et al., 2012). A role for Akt in pancreatic cancer cell migration has not been previously documented; however, results provided within this study provide a strong case for Akt involvement. Phosphorylation at both Thr308 and Ser473 is required for full activation of Akt, and although within this study only Ser473 phosphorylation was investigated, it still provides evidence for a PI3K/PAK4/Akt axis involvement in PDAC cell migration. Akt has been shown to induce tumour metastasis through Twist1 phosphorylation-driven EMT via crosstalk between TGF-β and PI3K signalling (Xue et al., 2012) and activated Akt (shown by increased Ser473 phosphorylation), was found to promote phosphorylation/activation of components important cell migration including actin filaments (Ho et al., 2011). Indeed, phosphorylation of Akt at both Thr308 and Ser473 was required for motility of lung endothelial cells

downstream of HGF, with pharmacological inhibition of PI3K via LY294002 treatment leading to reduced phosphorylation at both sites and decreased lamellipodia formation (Usatyuk et al., 2014). Furthermore, it has recently been reported that the Wnt family member, Wnt5A, promotes phosphorylation of Akt at Ser473 downstream of PI3K to promote osteosarcoma cell migration (Zhang et al., 2014a), supporting the hypothesis that PAK4 regulation of Akt phosphorylation at Ser473 may also be required for PDAC cell migration. Whether this is direct phosphorylation of via an intermediary requires further study, as does whether there is involvement of Thr308 phosphorylation.

However, what has been demonstrated here is the requirement for PAK4 in pancreatic cancer cell migration and invasion (summarised in **figure 7.1**). A novel interaction between PAK4 and p85α has provided evidence for the involvement of PAK4 in the PI3K pathway. It is thought that this could potentially be a mechanism to relieve PAK4 autoinhibition (similar to Src SH3), which then serves to promote phosphorylation of AKT at Ser473 and subsequently increase pancreatic cancer cell migration and invasion.


HGF
C-met
HGF
C-met
P
P
p85
Gab1
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P
K-ras
p110
*
p85
p110
PAK4
?
Ser473
P
AKT
Thr308?
P
Adhesions
Nucleus
Actin filaments
New adhesions
INCREASED
CELL
MIGRATION
AND
INVASION

Figure 7.1: Proposed model of RAS/PI3K/PAK4/AKT signalling module in pancreatic cancer. Data gathered throughout the course of this study provides evidence that PAK4 lies downstream of K-ras and PI3K to promote pancreatic cancer cell migration. PAK4 has been shown to bind to K-ras and to the p85α subunit of PI3K either directly via a PAK4PxxP/p85αSH3 domain interaction, or indirectly via the scaffold protein Gab1. It is thought that this signalling pathway is activated downstream of HGF-mediated c-Met activation and serves to increase phosphorylation of Akt at Ser473, which subsequently leads to increase both 2D migration and 3D invasion of pancreatic cancer cells. It has also been hypothesised that SH3-mediated binding of p85α to PAK4 (*) could serve to aid relief of PAK4 autoinhibition. Whether PAK4 phosphorylates Ser473 directly or through an intermediary (?) such as mTORC2 remains to be elucidated, as does the impact PAK4 may have on Akt phosphorylation at Thr308. However, it has been shown that PAK4 serves to increase pancreatic cancer migration in an Akt dependent manner, downstream of a novel interaction with PI3K.

*indicates potential binding that may aid in relief of PAK4 autoinhibition
? indicates possible intermediary protein between PAK4 and AKT

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